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Amphiphilic Peptide Interactions with Complex Biological Membranes

Effect of peptide properties on antimicrobial and anti-inflammatory effects

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Abstract
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With increasing problem of resistance development in bacteria against conventional antibiotics, as well as problems associated with diseases either triggered or enhanced by infection, there is an urgent need to identify new types of effective therapeutics for the treatment of infectious diseases and its consequences. Antimicrobial and anti-inflammatory peptides have attracted considerable interest as potential new antibiotics in this context. While antimicrobial function of such peptides is being increasingly understood demonstrated to be due to bacterial membrane disruption, the mechanisms of their anti-inflammatory function are poorly understood. Since bacterial membrane component lipopolysaccharide triggers inflammation, this thesis aims at clarifying importance of lipopolysaccharide (LPS)-peptide interactions while investigating possible modes of action of peptides exhibiting anti-inflammatory effect. Furthermore, effect of poly(ethylene)glycol (PEG)-conjugation was investigated to increase performance of such peptides.

Results presented in this thesis demonstrate that peptide-induced LPS- and lipid A binding/ scavenging is necessary but not sufficient criterium for anti-inflammatory effects of peptides. Furthermore, preferential binding to LPS over lipid membrane, as well as higher binding affinity to the lipid A moiety within LPS, are seen for these peptides. In addition, results demonstrate that apart from direct LPS scavenging, membrane-localized peptide-induced LPS scavenging seem to contribute partially to anti-inflammatory effect. Furthermore, fragmentation and densification of LPS aggregates, in turn dependent on the peptide secondary structure on LPS binding, as well as aromatic packing interactions, correlate to the anti-inflammatory effect, thus promoting peptide-induced packing transition in LPS aggregates as key for anti-inflammatory functionality. Thus, peptide-induced LPS aggregate disruption together with reduction of the negative charge of LPS suggests the importance of phagocytosis as an alternative to the inflammatory pathway, which needs to be further investigated. Furthermore, PEG conjugation of peptide results in strongly reduced toxicity at a cost of reduced antimicrobial activity but markedly retained anti-inflammatory effect.

Taken together, the results obtained in this work have demonstrated several key issues which need to be taken into consideration in the development of effective and selective anti-inflammatory peptide therapeutics for the treatment of severe Gram-negative bacterial infections.

Keywords: LPS, Antimicrobial, Peptide, Inflammation, Infections, Liposome, Binding, PEG

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Till min familj

"*Nothing in life is to be feared, it is only to be understood.*
Now is the time to understand more, so that we may fear less."

Marie Curie

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Singh S.**, Kasetty G., Schmidtchen A., Malmsten M. *Membrane and lipopolysaccharide interactions of C-terminal peptides from S1 peptidases*. Biochimica et Biophysica Acta, (2012), *1818*, 2244–2251

II **Singh S.**, Kalle M., Papareddy P., Schmidtchen A., Malmsten M. *Lipopolysaccharide interactions of C-terminal peptides from human thrombin*. Biomacromolecules, (2013), *14*, 1482–1492.

III **Singh S.**, Papareddy P., Kalle M., Schmidtchen A., Malmsten M. *Effects of linear amphiphilicity on membrane interactions of C-terminal thrombin peptides*. RSC Advances, (2014), *4*, 37582–37591.

IV **Singh S.**, Papareddy P., Kalle M., Schmidtchen A., Malmsten M. *Importance of lipopolysaccharide aggregate disruption for the anti-endotoxic effects of heparin cofactor II peptides*. Biochimica et Biophysica Acta, (2013), *1828*, 2709–2719.

V **Singh S.**, Papareddy P., Mörgelin M., Schmidtchen A., Malmsten M. *Effects of PEGylation on membrane and lipopolysaccharide interactions of host defense peptides*. Biomacromolecules, (2014), *15*, 1337–1345.

VI Datta A., Bhattacharyya D., **Singh S.**, Ghosh A., Schmidtchen A., Malmsten M., Bhunia A. *Role of aromatic amino acids in lipopolysaccharide and membrane interactions of antimicrobial peptides for use in plant disease control.* Manuscript submitted

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I was highly involved in the planning, study design, experimental work and data analysis of Paper II, III and IV. I was partly involved in the planning, study design, experimental work, data analysis and writing of Paper I, V and VI. I did not contribute to any larger extent to the biological data included in above papers.

Additional papers not included in this thesis

VII Duong D.T., **Singh S.**, Bagheri M., Verma N.K., Schmidtchen A., Malmsten M. *Pronounced peptide selectivity for melanoma through tryptophan end-tagging. Sci. Rep.* (2016) Manuscript accepted

VIII Papareddy P., Kalle M., **Singh S.**, Mörgelin M., Schmidtchen A., Malmsten M. *An antimicrobial helix A-derived peptide of heparin cofactor II blocks endotoxin responses in vivo* Biochimica et Biophysica Acta, (2014), *1838*, 1225-1234.

Contents

Abbreviations

AMP	Antimicrobial peptide
CD	Circular dichroism
DPI	Dual polarization interferometry
DLS	Dynamic light scattering
NTA	Nanoparticle tracking analysis
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
E. coli	*Escherichia coli*
S. aureus	*Staphylococcus aureus*
DOPE	1,2-dioleoyl -*sn*-glycero-3-phosphoethanolamine
DOPG	1,2-dioleoyl -*sn*-glycero-3-phosphoglycerol
DOPC	1,2-dioleoyl -*sn*-glycero-3-phosphocholine
PC	Phosphatidylcholine
PS	Phosphatidylserine
PI	Phosphatidylinositol
SM	Sphingomyelin
PG	Phosphatidylglycerol
DPG	Diphosphatidylglycerol
MDR	Multi-drug-resistance
p	Parallel
s	Perpendicular
TEA	Triethylamine
TM	Transverse magnetic
TE	Transverse electric
Δn_f	Birefringence
PCS	Photon correlation spectroscopy
PEG	Poly(ethylene)glycol
TLR4	Toll-like receptor 4

One-letter abbreviations of amino acids used in the thesis

Cationic

R	Arginine
L	Lysine
H	Histidine

Anionic

D	Aspartic acid
E	Glutamic acid

Uncharged/Polar

N	Asparagine
Q	Glutamine
G	Glycine
S	Serine
T	Threonine

Hydrophobic

F	Phenylalanine
W	Tryptophan
L	Leucine
I	Isoleucine
A	Alanine
V	Valine
Y	Tyrosine

Introduction

Due to increasing occurrence of multidrug resistant bacteria[1-3], there is an urgent need for novel infection therapeutics. In this context, new biologically active macromolecules, especially proteins and peptides, are currently receiving much attention in research due to recent rapid progress in genomics and proteomics, but also regarding advanced analytical methodologies. Among such peptides and proteins, antimicrobial peptides (AMPs) are interesting as potential sources of future therapeutics because of their broad-spectrum activities and different mechanisms of action compared to conventional antibiotics[4-7]. Currently, significant interest is directed towards AMPs, or host defense peptides, of endogenous origin, such as defensins, cathelicidins and histatins[8, 9]. Host defense peptides play a key role in innate immunity through rapid response against invading pathogens such as fungi, viruses and parasites. Such peptides form an evolutionarily conserved component of the innate immune response which provides direct broad-spectrum antimicrobial effects, but also a range of additional functionalities, including anti-inflammatory and immune modulating effects, as well as effects on chemotaxis and angiogenesis[10-13]. Ideally, such peptides should exert selectivity, achieved, e.g., through the fundamental differences between pathogen and host cells membranes, such as higher charge density for bacteria, or presence of cholesterol in host cell but not in bacteria membranes[14, 15], in order to combine potent antimicrobial effect with lower toxicity towards host cells. Among several classes of such peptides, a number of peptides derived from coagulation-related proteins have been identified as displaying interesting antimicrobial and anti-inflammatory properties, including C-terminal peptides from human thrombin[16, 17], other coagulation factors from the S1 peptidase family[18], and heparin cofactor II[19]. These peptides display potent anti-inflammatory and immune modulating effects as evidenced from both cell experiments on macrophages and results on animal models of septic shock induced by lipopolysaccharide (LPS)[16] and Gram-negative bacteria[20]. Due to this, such peptides are of interest as potential drugs against both acute (e.g., sepsis) and chronic (e.g., COPD) inflammation. While demonstrating promising biological effects, these studies did not clarify the mode of action of these peptides to display anti-inflammatory effect nor the role of LPS neutralization and how this affects antimicrobial activity. In order to investigate how these AMPs interact with

bacterial membrane and its components, studies on simplified model systems can be employed, which is the main focus of this thesis.

Antimicrobial peptides

Antimicrobial peptides (AMPs) constitute an important component of the innate immune system of multicellular organism, forming a first line of defense against invading pathogens, including bacteria, viruses, fungi, parasites, and even cancer cells[21-23]. AMPs have been identified from a number of sources, including plants[24], insects[25], and vertebrates[26], and until now over 2000 have been identified[27]. In humans, defensins and cathelicidins (LL-37) are the host abundant AMPs, found in higher concentration in pathogen-infected tissues such as skin, lungs and gastrointestinal tract[28, 29]. They are generally ≈10-40 amino acids long, having a molecular mass < 10 kDa, being net positively charged, and containing a considerable proportion of hydrophobic residues, frequently existing in pattern of 1-2 for every 3-4 residues, making them amphiphilic in nature [30]. AMPs have multiple targets, including interference with cell wall, DNA-, RNA- and protein synthesis as well as of enzymatic activity, but the main mode of action of their antimicrobial effect is disruption of bacterial membranes[31, 32]. Several mechanisms for this action have been proposed (*Figure 1*), including formation of barrel-stave or toroidal pores[31, 33], transient defects due to peptide translocation across the membrane[34, 35], membrane thinning causing membrane destabilization[36] as well as peptide-induced lipid segregation and phase transition[37, 38].

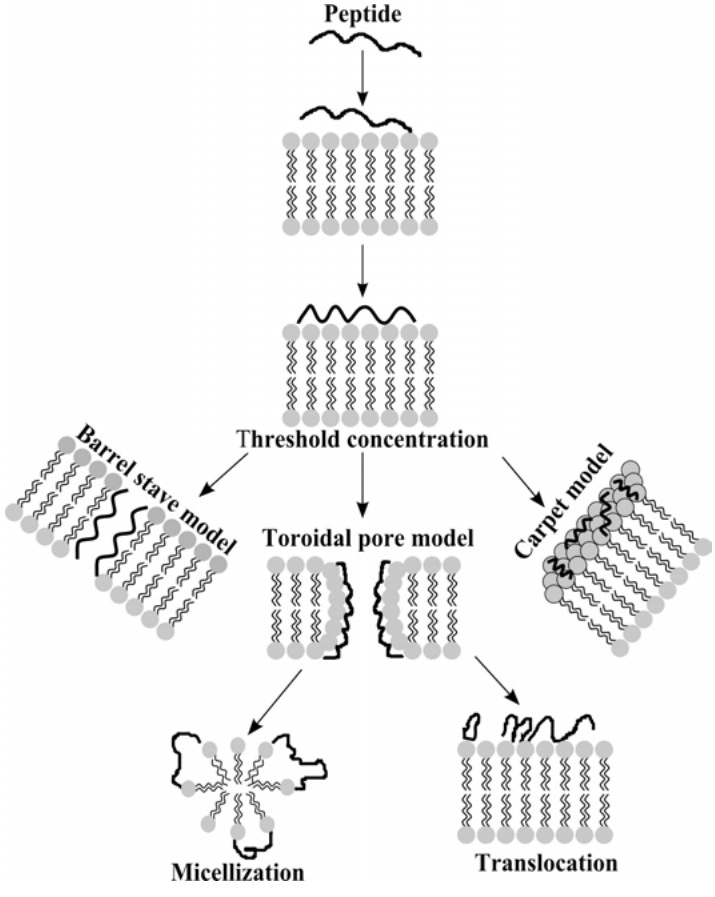


Figure 1. Schematic illustration of possible modes of action of AMP- induced membrane destabilization.

Structure-activity relationships (SAR)

AMPs can be classified into four groups based on their conformation adopted by peptides on interaction with bacterial membranes: α-helical peptides, β-sheet peptides, extended peptides and loop peptides[39, 40].

Helical peptides are the most abundantly distributed and widely studied groups of AMPs including, e.g., magainin, LL-37 and cecropin. These peptides display a distinct amphiphilic structure with about 50% hydrophobic residues, generally appearing in repeated patterns. Frequently, these peptides are unstructured in aqueous solution and adopt a helical conformation in membranes or membrane–mimicking environments[41, 42]. The α-helical peptides have been inferred to kill bacteria by creating

membrane defects in the bacterial membrane. Some α-helical peptides such as some magainins are thought to form toroidal pores to lyse the bacterial membranes[33].

In contrast to helical peptides, β-sheet AMPs such as β-defensins are cyclic molecules stabilized by disulfide bridges to form a well-defined β-strand. Subsequently, cysteine is also largely overexpressed in these peptides such as all defensins contain 6-8 highly conserved cysteines that result in three or four disulfide bonds[13]. It has been previously shown that presence of disulfide bonds plays an important role for the functionalities of such peptides, including increased resistance against (bacterial) proteolysis[43]. These AMPs display their antimicrobial activities through bacterial membrane disruption by inserting perpendicular into the lipid bilayer to form toroidal pores[44].

Finally, extended AMPs do not present a specific motif or conformation; instead they are predominantly rich in specific amino acids such as proline, tryptophan, arginine and histidine. Examples of such peptides include, e.g., indolicidin, a tryptophan/proline dominated extended peptide, as well as Bac 5 and Bac 7, which are proline/arginine-rich peptides, and histatins, which are histidine-enriched peptides[45, 46]. It has been speculated that extended AMPs are not only active against bacterial membrane but also exert antimicrobial activities by penetrating across the membranes and interacting with bacterial proteins[44].

Factors affecting antimicrobial activity

AMPs are characterized by a set of biophysical factors which determine their selectivity. In this respect, net positively charged AMPs is responsible for initial interaction of the AMP with negatively charged bacterial membranes due to over-representation of cationic lysine/arginine/histidine residues and the sparsity of negatively charged amino acids, such as aspartic or glutamic acid[47, 48]. Since bacterial membranes are anionic, peptide-induced bacterial membrane disruption generally increases with peptide net positive charge. However, increasing the charge beyond an upper limit does not increase the lytic activity further due to strong electrostatic interaction between the peptide and lipid head groups which precludes structural rearrangement and insertion into the deeper core of membranes[49].

Together with this, hydrophobicity is another important physicochemical characteristic of AMPs that can modulate the antimicrobial activity and specificity through governing peptide permeabilization into the lipid bilayers[15, 50]. Furthermore, hydrophobic interaction helps to maintain antimicrobial activity for highly charged peptides at physiological condition through avoiding electrostatic screening-based inactivation[21, 32]. However, highly hydrophobic peptides may display a loss of antimicrobial activity, e.g., resulting from peptide aggregation[51], and more importantly, increased

toxicity towards mammalian cells[52]. Thus, a good balance is needed between electrostatic and hydrophobic interactions to achieve higher antimicrobial potency without losing selectivity.

In addition, peptide binding to, and destabilization of, lipid membranes is expected to decrease with decreasing peptide length due to increased penalty per amino acid on adsorption[53]. Finally, peptide secondary structure has been found to strongly affect peptide-induced membrane rupture[54]. Consequently, a pronounced helix formation on membrane interaction provides an additional driving force for peptide binding to lipid membranes. Thus, a reduction in helix-related amphiphilicity through selected D-amino acid substitutions results in reduced membrane disruption as well as decreased peptide-induced liposome leakage and bacterial killing[55, 56]. In parallel, decreasing AMP helicity generally reduces cytotoxicity of AMPs[31], however such toxicity reduction may ultimately result in reduction or elimination of antimicrobial effect, illustrating that a balance is needed for optimal activity of such peptides.

Bacterial membranes

AMPs selectivity is a primary challenge in the identification of AMPs for future clinical use, so that they are potent against bacteria but at the same time non-toxic to human cells. The basis of selectivity is due to difference between bacterial membranes and membranes of mammalian cells. For example, cholesterol is a fundamental part of mammalian cell membranes, while it is absent in bacterial membranes[14]. There are also considerable differences in phospholipid composition between bacterial and human cell membranes. In contrast to bacterial membranes, mammalian cytoplasmic membranes are rich in zwitterionic lipids, primarily PC (phosphatidylcholine), PE (phosphatidylethanolamine), and SM (sphingomyelin) and to a lesser extent the anionic PS (phosphatidylserine) and PI (phosphatidylinositol). The latter two are localized to the inner leaflet[57]. This results in an electropotential of mammalian cells that is substantially lower than that of bacteria[57, 58].

Bacteria are classified according to the Gram-staining technique as being either Gram-negative or Gram-positive bacteria, a differentiation based on their cell wall properties (*Figure 2*). A major difference between them is that Gram–positive bacteria have only one lipid membrane, the cytoplasmic membrane that surrounds the cell, while Gram-negative bacteria are characterized by the presence of two distinct membranes, i.e., the inner/cytoplasmic membrane and an outer membrane. Both bacteria types have a peptidoglycan layer on the outside of the cytoplasmic membrane. This peptidoglycan layer is typically a few nanometers thick for Gram-negative bacteria, but much thicker (30-100 nm) and containing many layers,

in Gram-positive bacteria[59, 60]. Another difference is that both types of bacteria contain different polymeric chain in their membranes although both have phosphate groups in common and are negatively charged. In case of Gram-positive bacteria, these long anionic polymeric chains are lipoteichoic acids (LTA) that are imbedded in the cytoplasmic membrane, while, in the case of Gram-negative bacteria, the outer membrane is covered by lipopolysaccharide (LPS).

In contrast to mammalian cells, the cytoplasmic membrane in bacteria is highly negatively charged due to presence of PG (phosphatidylglycerol) as well as DPG (diphosphatidylglycerol) (also known as cardiolipin). For most bacteria, the predominant phospholipid is PE. In general, Gram-negative bacteria have a higher content of PE than Gram-positive bacteria[61].

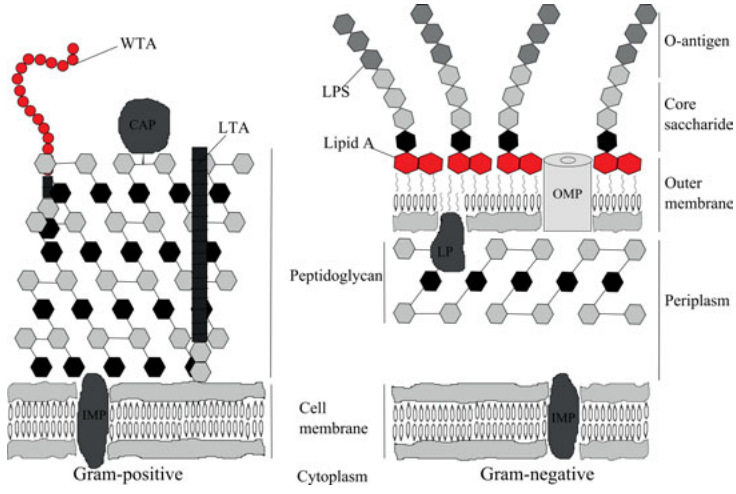


Figure 2. Schematic illustration of the membranes of Gram-positive and Gram-negative bacteria. In addition, different membrane components (CAP, covalently attached protein; IMP, integral membrane protein; OMP, outer membrane protein; LPS, lipopolysaccharide; LTA, lipoteichoic acid; WTA, wall teichoic acid) are also shown.

Lipopolysaccharide

In Gram-negative bacteria, LPS (also referred to as endotoxin) constitutes a major molecular component of the outer leaflet of the outer bacterial membrane, which plays an important role in Gram-negative infections and in sepsis[62]. It covers more than 70% of the cell surface of its outer leaflet[63], serving as a physical barrier and protecting bacteria from their surroundings. LPS is recognized by the immune system as a marker for bacterial infection and triggered inflammatory responses, causing endotoxic shock in extreme

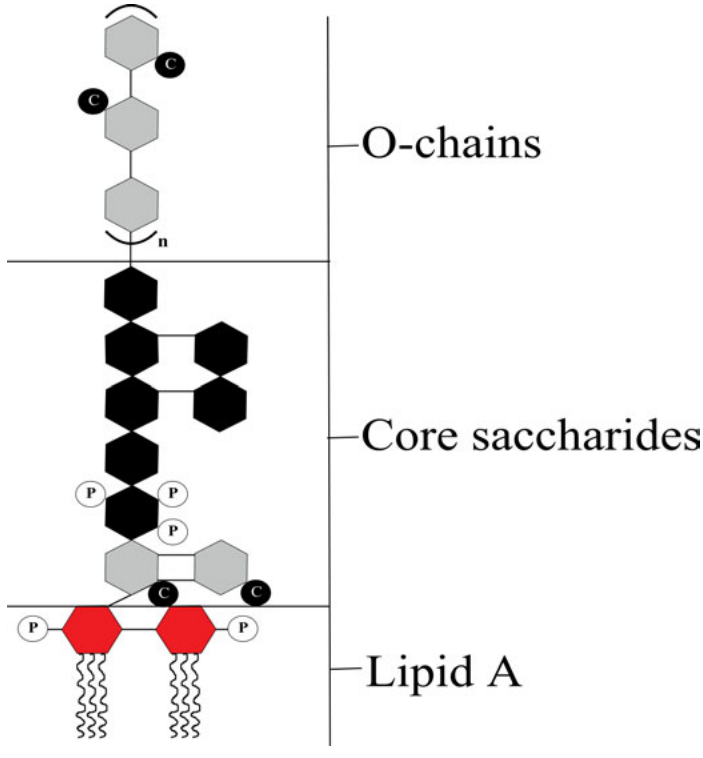
cases[64]. LPS consists of a preserved hydrophobic lipid component (lipid A), a short oligosaccharide (R-core), and an outer polysaccharide region (O-antigen)[64, 65] (*Figure 3*). The characteristic structural features of lipid A, mainly its two phosphate groups and its two acyloxyacyl moieties, have been thought necessary to trigger the inflammatory response in human cells[66], although other studies have demonstrated the situation to be more complex than this, e.g., through finding that LPS inflammatory triggering depends on LPS aggregation[67]. LPS is anchored to lipid A in the membrane and contains negative charge through phosphate and carboxyl groups. Because of triggering inflammation, the interaction of LPS with LPS binding AMPs has attracted attention[68].

Lipoteichoic acid

Similar to LPS, teichoic acid represents the major virulence factor of Gram-positive bacteria. They are long, anionic cell surface polymers which are composed of glycerol phosphate, glucosyl phosphate or ribitol phosphate repeats[60]. There are two types of teichoic acids: wall teichoic acids (WTAs) and lipoteichoic acids (LTAs). WTAs are coupled to peptidoglycan via a phosphodiester linkage whereas LTA are anchored to the head groups of membrane lipids[69]. Thus, they extend from the cell surface into the peptidoglycan layers rather than through and beyond. Collectively, these polymers can account for over 60% of the mass[69] of the Gram-positive cell wall, contributing at large extent to the envelope structure and function.

LTA are composed of polyglycerol phosphate (polyGroP) chains that are often functionalized with a sugar moiety[70]. Interestingly, LTA shares with lipopolysaccharide many of its pathogenic properties. It is recognized by the immune system as a sign of bacterial infection and triggered inflammatory cascades through binding to lipopolysaccharide-binding protein, recognized by CD14 and Toll-like receptors (TLR) resulting in induced secretion of proinflammatory cytokines.

(a)



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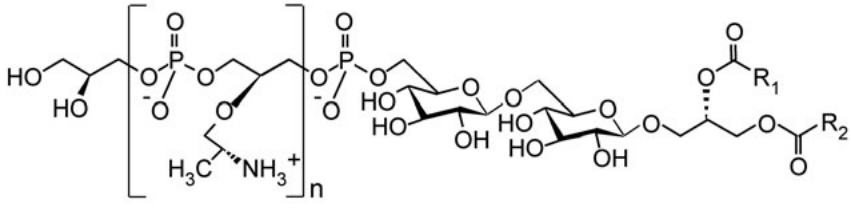


Figure 3. Schematic structure of LPS (a) in Gram-negative bacteria and LTA (b) in Gram-positive bacteria.

Inflammation

LPS- induced Inflammation

As part of host defense system, monocytes and macrophages play important roles against invading pathogens. In general, upon pathogen recognition, a series of inflammatory response is rapidly induced along with production of various cytokines, such as TNF-α, IL-6 and IL-12, as well as other biologically active substances, which subsequently permits effective clearance of the pathogen[71]. The recognition of pathogens and their pathogen-related molecular reactions depend on a diverse set of receptors[72] such as TLR4 (Toll-like receptor 4) which recognizes lipid A component of LPS[73]. TLR4 belongs to a group of innate immunity receptors that contains a large extracellular domain of leucine-rich repeats, a single *trans*-membrane segment and a smaller cytoplasmic signaling region. In the classical activation pathway, the lipid A moiety of LPS binds to acute-phase plasma LPS-binding protein (LBP), a 60 kD serum glycoprotein that forms a high-affinity stoichiometric complexes with LPS[74]. This LPS-LBP complex is further recognized by CD14, a differentiation antigen found at the cell surface of monocytes/macrophages, leading to interaction with the TLR4/myeloid differentiation protein-2 (MD2) complex[68] (*Figure 4*). MD2 is known as TLR4-binding protein that is required for the function of TLR4 [75] and enhances the biological activity of LPS/LBP complex in TLR4. Furthermore, LPS/LBP complex binding to TLR4/MD2 associate cause TLR4 receptor dimerization[76], crucial for its functionality in inducing intracellular signaling, leading to up-regulation of NF-κB and other proinflammatory cytokines. In general, these functions are desirable for clearing local infections as part of the natural host defense, but continuous cell stimulation might result in an uncontrolled host response leading to tissue damage and multiple organ failure as seen in severe sepsis[77].

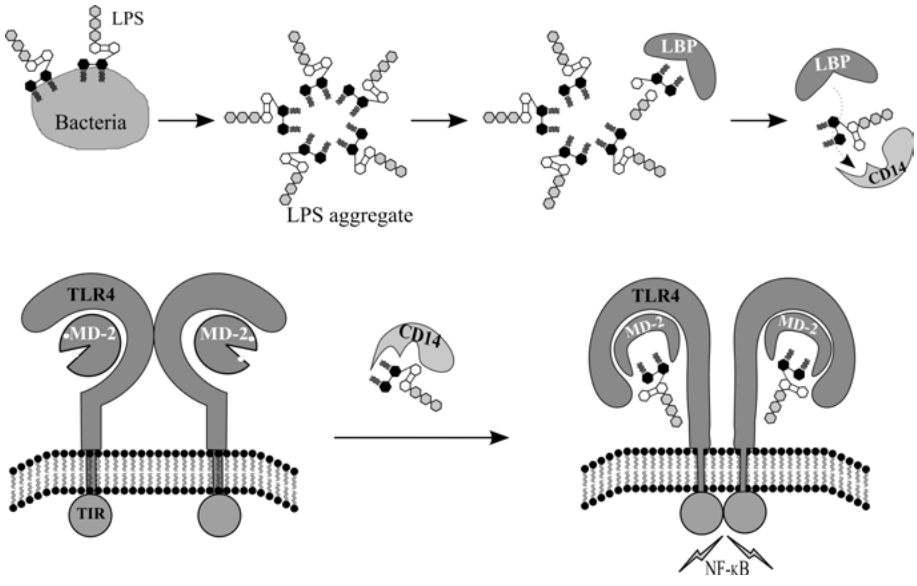


Figure 4. Schematic illustration of the classical pathway of LPS-induced NF-κB activation reaction in macrophages.

Peptide role in inflammation

Besides natural host defense mechanism produced by immune cells, host defense peptides (HDPs) play an important role in innate immune system by facilitating clearance of invading pathogens as well as boosting infection-resolving immunity[39]. Consequently, they have attracted attention as potential anti-infective therapeutics due to their ability to not only kill bacteria, but also modulate a verity of immune responses[32]. For example, studies based on various cationic HDPs have shown that peptides binding to LPS may provide an alternative binding site, thus block the subsequent LPS-LBP binding interaction, resulting in suppression of TNF- α production by macrophages[78]. Furthermore, HDPs can reduce proinflammatory responses, and organ dysfunction seen in mouse models of septic shock and bacterial infections[79, 80]. Apart from LPS neutralization, HDPs have been speculated to be able to interfere with the TLR4 recognition system by disturbing the local membrane surroundings of the receptor, thus modifying its activation state, leading to reduction of cytokine production and alteration of the inflammatory response[81]. Furthermore, immunomodulatory activity of HDPs can manipulate monocyte responses, thereby inhibiting release of proinflammatory cytokines as shown by LL-37[82]. In addition to this, the thrombin-derived C-terminal peptide GKY25 was shown to inhibit LPS–induced inflammatory response through LPS binding and interaction with monocytes/macrophages as well as interfering with TLR4/MD2 dimerization[83].

Motivation

Antimicrobial peptides (AMPs) play a crucial role in host defense displaying fast and broad spectrum antimicrobial effects as well as anti-inflammatory effects. While the antimicrobial effects of such peptides are becoming increasingly understood, the mechanisms underlying anti-inflammatory properties of host defense peptides remain unclear. Due to inflammatory properties of lipopolysaccharide (LPS) in Gram-negative bacteria and lipoteichoic acid (LTA) in Gram-positive bacteria, various approaches have been used to investigate peptide interactions with these inflammatory (lipo)polysaccharides. While, structure-activity-relationship investigations have been extensively reported with regard to AMP-membrane interactions and their resulting antimicrobial effects, systematic studies on how peptide physiochemical properties such as length, charge, hydrophobicity and secondary structure affect peptide-LPS interaction and the resulting anti-inflammatory effects are more scarcely reported in literature. For example, there has been limited work done regarding the relative importance of AMP binding to LPS, its "endotoxic principle" lipid A component and lipid membranes. Furthermore, peptide-induced LPS binding could be envisioned to anti-inflammatory effects through several different mechanisms, as a growing number of studies have demonstrated LPS binding to be necessary, but not sufficient criteria for anti-inflammatory effects. In order to address this, a series of investigations have been carried out regarding peptide binding to LPS, lipid A, and phospholipid membranes, and how this contributes to peptide anti-inflammatory effect.

Aim of the thesis

The overall aim of this thesis was to clarify the mechanisms of action of anti-inflammatory peptides through investigating binding of these peptides to Gram-negative bacteria and its membrane components, as well as their significance for anti-inflammatory and antimicrobial effects. In doing so, peptide interactions with lipid membrane and non-lipid membrane components were studied, including:

- Peptide-lipopolysaccharide binding and its correlation to anti-inflammatory effects
- Peptide-Lipid A binding and its contribution to anti-inflammatory effects
- Effects of peptide-LPS binding on LPS aggregate disruption, and consequences thereof on anti-inflammatory properties
- Detailed structural studies of peptide/LPS complexes
- Peptide-induced membrane scavenging as an anti-inflammatory mechanism
- Effects of PEGylation on peptide anti-inflammatory properties
- Peptide binding to, and disordering of, supported lipid bilayers as well as their consequences on antimicrobial effects

In addition, the thesis aimed to provide a deeper understanding of peptide properties such as the effect of peptide length, composition, charge and secondary structure on antimicrobial and anti-inflammatory effect.

Experimental techniques

Ellipsometry

Ellipsometry is an optical technique, which can be used for studying interfacial systems and processes, such as lipid bilayer formation or peptide adsorption to supported lipid bilayers[84-86] . This requires only a low–power light source which does not induce any photo damage in most studies, which makes ellipsometry a convenient tool for in situ studies. This technique is based on measurements of the change in polarization of light reflected from a surface.

Polarized light can be divided into a parallel (p) and a perpendicular (s) component in relation to plane of incidence. Upon reflection at a surface, both phase and amplitude of reflected light changes in a distinct manner depending on the optical properties of the surface. An ellipsometry measurement allows the quantification of these changes in term of the optical angles Δ and ψ, where the change in amplitude [E] (the ratio between the reflected (r) and incident (i) light for the parallel and perpendicular components, which can be translated into the reflection coefficient (R)) described by tan ψ, and Δ, which describes the corresponding phase difference (δ) between reflected and incident light.

$$\tan\Psi = \frac{R_p}{R_s} \tag{1}$$

$$\Delta = (\delta_p^r - \delta_s^r) - (\delta_p^i - \delta_s^i) \tag{2}$$

The overall ellipsometric response from measurement in presence of adsorbed film on the surface can be written as

$$\tan\psi\, e^{i\Delta} = \rho\,(n_0, n_1, n_2, d_1, \varphi_0, \lambda) \tag{3}$$

where λ is wavelength of light, φ_0 is angle of incidence, n_1 and d_1 the refractive index and thickness of the film, respectively. n_0 and n_2 refers to the refractive index of the bulk and the pure substrate, respectively (*Figure 5*).

From this model, two parameters n_1 and d_1 can be obtained from ψ and Δ. The value of the refractive index and thickness of the adsorbed layer can be used to calculate the adsorbed amount (Γ) according to[87]:

$$\Gamma = d_1 \frac{(n_1 - n_0)}{dn/dc} \tag{4}$$

where dn/dc is refractive index increment of the adsorbing component.

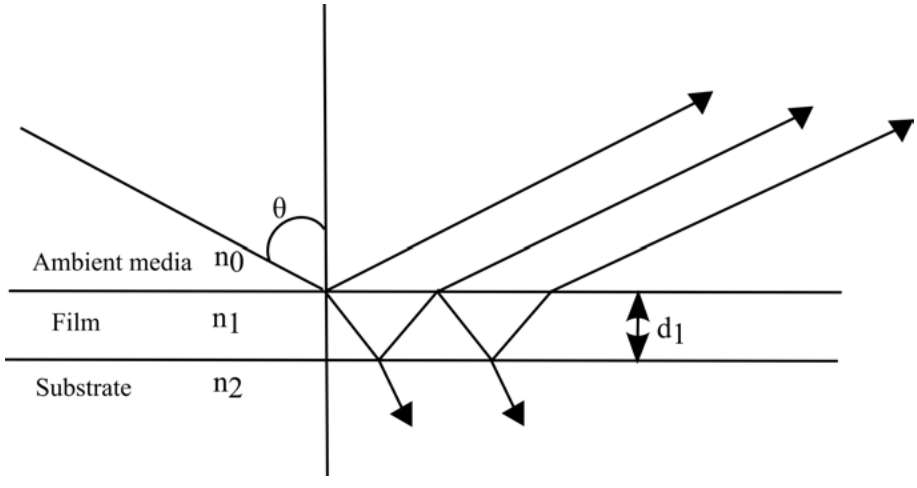


Figure 5. Reflection of polarized light at film-covered surface.

Substrates

The most commonly used substrate in ellipsometry is silica,[88] due to smoothness, optical properties, and the versatility of silicon oxide (SiO_2) layers formed under various conditions[89]. In this work, silicon slides were oxidized prior to use, forming a SiO_2 layer with a thickness of 30 nm, thus avoiding instability caused by spontaneous oxidation of silicon during measurements. This oxide layer furthermore enhances the separation of n and d. The substrates were cleaned by boiling in basic peroxide solution for removing organics, followed by acidic peroxide solution for removing inorganic contamination [90]. Before use, the substrates were further treated by plasma cleaner, resulting in surfaces with a contact angle less than 10^o. These surfaces were further silanized under vacuum to prepare methylated

silica surfaces (surface potential -40 mV, contact angle 90^{o})[91] for deposition of *E. coli* LPS and lipid A.

Experimental setup

Schematic description of the instrumental set up is shown in *Figure 6*. A typical ellipsometer requires a light source, a polarizer, a compensator, an analyzer and a photodetector. The light source, a Nd:YAG laser, generates a light beam which is converted to linearly polarized light after passing through the polarizer. After this, a change in phase of light obtained through the use of compensator and elliptically polarized light is produced before incidence on the sample. When polarized light of a particular ellipticity hits the substrate under investigation, the state of polarization changes once more and the light will become linearly polarized after reflection. The state of polarization of this reflected light is easily determined by a photodetector.

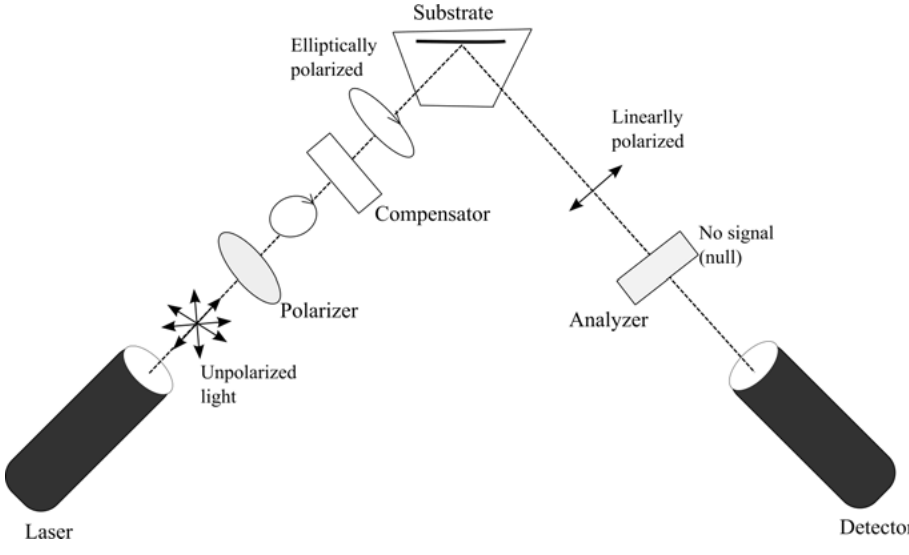


Figure 6. Schematic diagram of the null-ellipsometer setup showing different instrumental components along with change in polarization of the light (circular, elliptical, or linear) at different positions.

In adsorption studies, the most commonly employed approach is null ellipsometry setup which has been used throughout all the papers included in this thesis. The basis for null ellipsometry is that minimum light intensity should reach the photomultiplier, which is achieved by moving the positions of the polarizer and analyzer while keeping the compensator fixed at 45^{o}, until minimum light intensity is recorded.

Bilayer deposition

The phospholipid bilayers were deposited directly on silica surfaces by following two strategies: mixed micelle- solution or liposomes, depending on the lipid composition.

Zwitterionic supported bilayers were deposited on silica substrate by using mixed micelle- solution[92] approach. For this purpose, lipids (DOPC and cholesterol) were solubilized by a non-ionic surfactant DDM (*n*-dodecyl-β-D-maltoside) to form a mixed micelle solution. After addition of mixed micellar solution, adsorption was allowed to reach the equilibrium. Once adsorption equilibrium was attained, the cuvette was rinsed in order to solubilize micelle by dilution due to removal of surfactant and unadsorbed lipids. This procedure was repeated with decreasing micelle concentrations until the substrate is saturated with lipids and a stable, densely packed lipid bilayer is formed as shown in *Figure 7a*. However, mixed –micelle approach results incomplete adsorption for formation of anionic supported bilayers. Liposome adsorption approach was used for this purpose. Silica substrate was precoated with positively charged polylysine (at very low amount and under low ionic strength conditions in order to obtain a flat polylysine layer[93]) prior to lipid addition in order to avoid peptide adsorption directly to the silica through possible defects in the bilayer. Liposomes were added after removal of unadsorbed polylysine. Liposomes adsorption was allowed to stabilize as shown in *Figure 7b*.

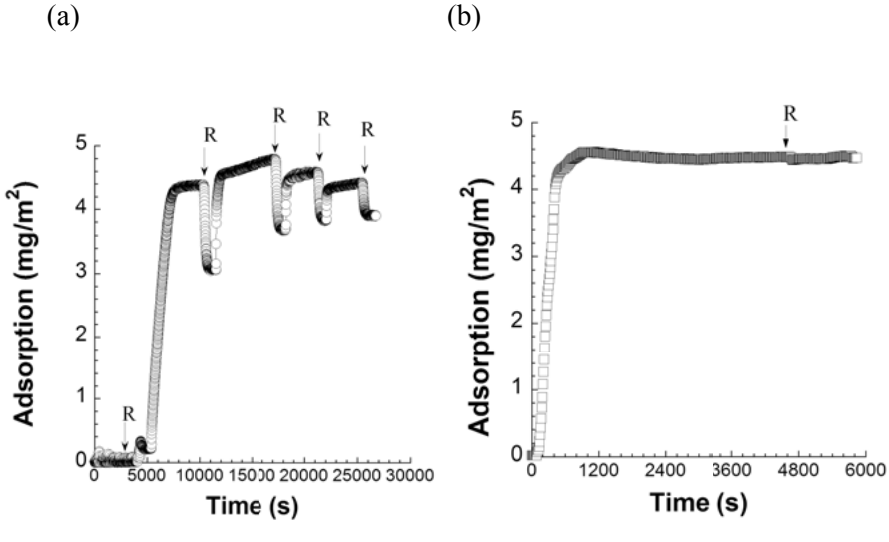


Figure 7. Formation of supported DOPC/cholesterol bilayers by using mixed - micelle approach (a) and DOPE/DOPG lipid bilayer (b) by liposomes adsorption. R indicates where rinsing starts.

LPS/ Lipid A deposition

E. coli LPS was used for this purpose and stock solution was obtained by dissolving LPS in milliQ water at room temperature.

LPS- coated surfaces were obtained by adsorbing *E. coli* LPS to methylated silica surfaces (surface potential -40 mV, contact angle 90° [91]) from LPS stock solution in water. This LPS stock solution was added to the cuvette at a concentration of 0.4 mg/ml and allowed to stabilize over a time period of 2 h. This results in a hydrophobically driven LPS adsorption reaching a plateau in the LPS adsorption isotherm as shown in *Figure 8a-b*. Non-adsorbed LPS was removed by rinsing.

For lipid A deposition, *E. coli* Lipid A was solubilized in 0.25 wt % triethylamine (TEA) under vigorous vortexing and heating the solution to 60°C for 10 minutes[94]. Lipid A was adsorbed at methylated silica surfaces at a concentration of 0.4 mg/ml in the cuvette and allowed to stabilize as shown in *Figure 8c*. Non-adsorbed lipid A was subsequently removed by rinsing.

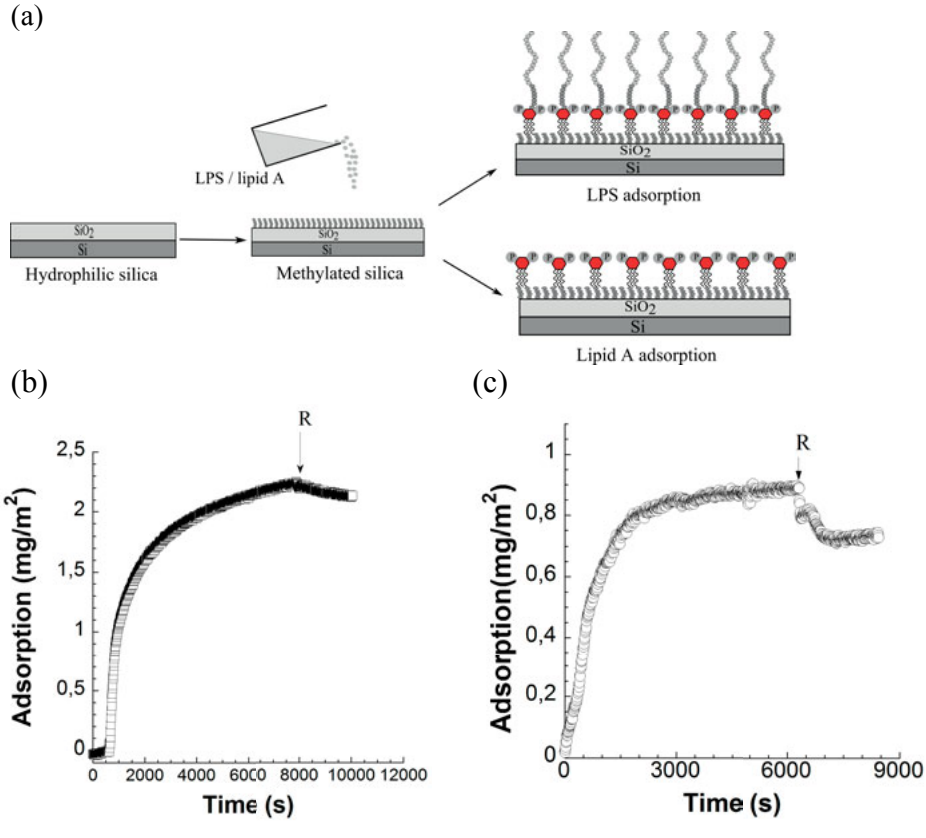


Figure 8. (a) Schematic presentation of LPS and lipid A deposition on hydrophobic silica surface. Representative LPS adsorption kinetics (b) and lipid A deposition kinetics (c) at methylated silica. R denotes when rinsing starts.

Liposomes leakage studies

Model dye-incorporated liposomes were prepared by adding fluorescent dye 5(6)-carboxyfluorescein (CF) to a dry lipid film of lipid compositions during the re-hydration step. Unilamellar liposomes were formed by subjecting the lipid mixture to freeze-thaw cycle, consisting of alternately freezing the solution in liquid nitrogen and heating to 60^{o} C, followed by multiple extrusions through a 100 nm membrane filter. Untrapped CF was removed by gel filtration by running the sample through the column.

Peptide–induced leakage from liposomes was investigated by fluorescence spectroscopy to study peptide-induced destabilization of the liposomes membrane. As mentioned above, liposomes were incorporated with a fluorescent dye, CF, a self-quenching at the high concentrations used inside the liposomes. Due to peptide-induced membrane destabilization CF is released from the liposomes interior. Leakage from liposomes was studied by monitoring reduction of CF self-quenching upon release from the liposomes interior. The emitted fluorescence from the liposome was followed at 520 nm. For leakage experiment in the presence of LPS, 0.02 mg/ml LPS was first added to the above liposome dispersion (which did not cause liposome leakage in itself), after which peptide was added and leakage monitored as a function of time. An absolute leakage scale was obtained by disrupting the liposomes at the end of each experiment through addition of Triton X-100. Measurements were performed at 37 °C.

Dual Polarization Interferometry

Dual polarization interferometry (DPI) method can be used to study surfactants, polymers and biological films, including lipid bilayers[95].

DPI is based on dual slab waveguide, consisting of an upper experimental waveguide (supporting the bilayer) and a lower reference waveguide[96, 97]. A schematic description of the instrumental setup is shown in *Figure 9*. The laser beam used in DPI can be divided into two polarizations modes, the transverse magnetic (TM) and transverse electric (TE). In the absence of a surface coating, light travels through both experimental and reference waveguides in phase. On peptide/lipid adsorption, on the other hand, light exits the two waveguides out of phase and produces interference fringe patterns which is recorded by a camera, depending on the optical properties of the adsorbed layer.

Although disordered phospholipids are often assumed to be optically isotropic, which is a good accurate approximation for unsaturated and disorganized phospholipid bilayers, also these actually display some optical birefringence which can be measured by this sensitive technique. The Δn_f obtained from difference in refractive indices for the TM and TE waveguide

modes (assuming the bilayer thickness to be constant) reflects alignment of lipid molecules in the bilayer[98]. The value of Δn_f increases when a lipid layer organizes from less ordered to more ordered and defined state. Conversely, it decreases on reducing alignment of the lipid molecules on insertion of antimicrobial peptides into the lipid bilayer.

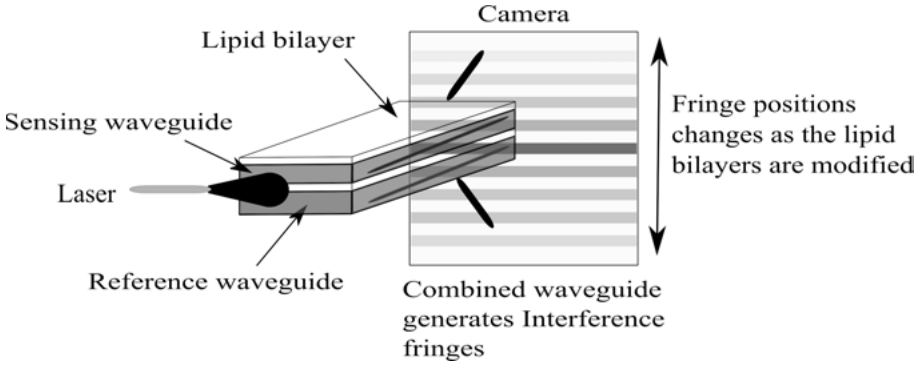


Figure 9. Schematic presentation of dual polarization interferometry.

Circular dichroism

Circular dichroism (CD) was used to investigate peptide conformation in solution and when bound to either liposomes or LPS (Paper I-VI). CD measures the differential adsorption of left– and right-handed circularly polarized light, generated by optically active chiral groups, and provides an information of the average conformation of peptides[99]. Different secondary structures show different characteristic CD spectra. Therefore, CD spectra for an unknown peptide structure can be compared to reference spectra of known secondary structure in order to determine the fraction of peptide conformation. In the present work, the fraction of α- helical conformation (X_α) was calculated from the following equation,

(5)

$$X\alpha = \frac{A - Ac}{A\alpha - Ac}$$

where A is the recorded CD signal at 225 nm and A_c and A_α is the CD signal at 225 nm for a reference peptide at 100% random coil and 100% α-helix conformation, respectively[100]. Although 190-250 nm is generally used for

secondary structure predictions, the signal is often poor below 200 nm, and particularly so in the presence of colloidal-sized particles, such as liposomes.

Dynamic light scattering (DLS)

In order to characterize the LPS-peptide aggregate size and also to investigate whether peptides cause liposome aggregation or coalescence, we used dynamic light scattering (DLS), also known as photon correlation spectroscopy (PCS). This technique relies on intensity fluctuations of scattered light due to random motions of particles. These fluctuations are dependent on the size of particles, thus by measuring intensity autocorrelation over time, the diffusion constant (D) of the particles can be obtained. The diffusion constant is related to the hydrodynamic radius (R_h) of the particles according to the Stokes- Einstein equation[101]:

$$D = \frac{k_B T}{6\pi\eta R_h} \tag{6}$$

where k_B is Boltzmann constant, T is the temperature, η is the viscosity of the medium.

Furthermore, liposomes and peptide/LPS aggregates were characterized by their zeta-potential (ζ) through measuring their electrophoretic mobility, i.e., particle velocity when an electric field is applied across the solution. The electrophoretic mobility (u) is obtained by detecting the light scattered by the moving particles. Later, z-potential (ζ) was calculated from electrophoretic mobility of particles by considering the Helmholz-Smoluchowski equation[101]:

$$\zeta = \frac{\eta}{\varepsilon\varepsilon_0}\, u \tag{7}$$

where η is the viscosity of the solution, ε is the dielectric constant and ε_0 is the dielectric constant of vacuum.

Nanoparticle tracking analysis (NTA)

Nanoparticle tracking analysis is a powerful characterization technique for analyzing particles of nanometer scale (~ 30 to 1000 nm), with lower detection limit depending on refractive index of the nanoparticles investigated. This technique is based on a laser illuminating microscope with a high sensitivity charge-coupled device (CCD) camera which captures scattered light of particles undergoing Brownian motion when in solution.

NTA records video of nanoparticles in order to track the Brownian motion of each individual particle to yield independent size measurements[102, 103]. Individual nanoparticles moving under Brownian motion are identified and tracked, followed by calculation of the mean hydrodynamic diameter (R_h) according to the Stokes-Einstein equation:

$$(\bar{x}, y)^2 = \frac{2k_B T}{3\pi\eta R_h} \tag{8}$$

where k_B is Boltzmann constant and $(\bar{x}, y)^2$ is the mean square speed of the particles at temperature T, in a solution of viscosity η, with a hydrodynamic radius of R_h.

NTA enables individual particle tracking, particle visualization and appropriate particle concentration determination which are characteristic features of NTA, affords a more accurate number size distribution analysis of particles compared to dynamic light scattering. This technique is used to characterize accurately the size distribution of both monodisperse and polydisperse samples whereas DLS measures monodisperse samples more accurately compared to polydisperse samples[104].

Tryptophan fluorescence spectra

Due to its abundance in antimicrobial peptides, as well as its environment-sensitive fluorescence, the latter is often used for monitoring peptide insertion into lipid membranes. Tryptophan results higher fluorescent signal mainly due to its emission wavelength shift in response to the change in polarity of its surrounding[105]. In general, if the emission maximum is less than 330 nm, this indicates that the tryptophan residue to be localized in a nonpolar environment, whereas emission maximum above this reports on a polar environment. Thus, tryptophan incorporation into membrane interior results a shift of emission maximum towards lower wavelengths, usually termed blue shift. The blue shift is observed in tryptophan-containing AMPs may be used to report on several different processes, such as peptide insertion and peptide aggregation.

Results and discussion

Peptide binding to LPS

Apart from lipid membrane binding, the antimicrobial and anti-endotoxic effects of AMPs depend on their interactions with non-lipid membrane component, notably lipopolysaccharide (LPS) in Gram-negative bacteria and lipoteichoic acid (LTA) in Gram-positive bacteria. In our first effort to address these effects (Paper I), a series of S1-derived amphiphilic peptides were used in order to investigate peptide binding to LPS and how it correlates to the anti-inflammatory effects of these peptides.

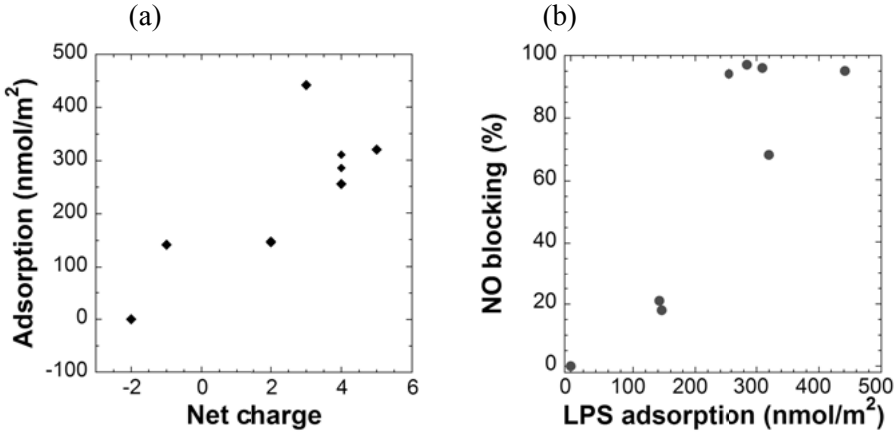


Figure 10. (a) Peptide binding to *E. coli* LPS and its correlation to peptide charge density. (b) Correlation between peptide adsorption to *E. coli* LPS and effect on LPS-induced NO production by macrophages. (Paper I)

Most of the peptides investigated bind to LPS, the extent on binding increasing with the peptide net charge (*Figure 10a*), but also mean hydrophobicity. This is logical considering the anionic nature of the LPS carbohydrate chain and the hydrophobicity introduced through its endotoxic principle "lipid A" moiety. Similarly, *Andrä et al.* reported on electrostatically driven LPS binding of NK-2, but also that hydrophobic interactions are important for efficient neutralization of the biological

activity of LPS[106]. In line with this, *Japelj et al.* found the importance of both electrostatic and hydrophobic effects on LF11 binding to LPS[107]. Furthermore, LPS binding was correlated to the ability of peptides to block NO production by macrophages and thus, anti-inflammatory activities of these peptides, as shown in *Figure 10b*. Peptides displaying extensive as well as to some extent binding to *E. coli* LPS display potent anti-inflammatory activity, while peptides with null LPS binding did not show any anti-inflammatory effects. Thus, LPS binding seems to be crucial requirement for the anti-inflammatory effect of these peptides. Quantitatively, however, it was found that the anti-inflammatory effects, while following the overall trend of LPS binding, did not scale perfectly with the amount of LPS binding (*Figure 10b*). Taken together, LPS binding was found to be a necessary, but not the sole, criterium of the anti-inflammatory effects of these peptides.

Table 1. *Primary structure and key properties of peptide investigated (Paper II).*

Peptide	Sequence	Z_{net}[1] *(pH 7.4)*	H[2]
GKY25	GKYGFYTHVFRLKKWIQKVIDQFGE	+3	-0.52
GKY25d	GKYG(dF)YTH(dV)FRL(dK)KWI(dQ)KVI(dD)QFGE	+3	-0.52
WFF25	WFFFYYLIIGGGVVTHQQRKKKKDE	+3	-0.52

[1]Z_{net}: net charge, [2]H mean hydrophobicity

In Paper II, thrombin-derived GKY25 peptide variants were chosen (*Table 1*) for investigating AMP binding to LPS and its hydrophobic lipid A moiety.

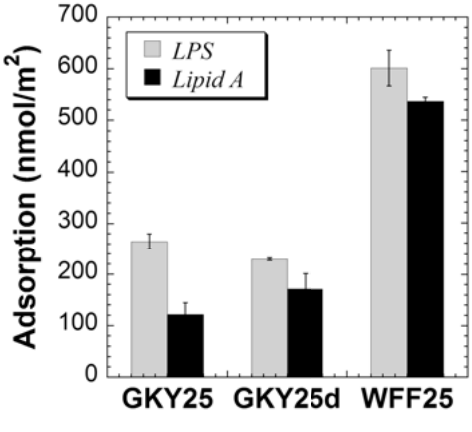


Figure 11. Peptide binding to *E. coli* LPS (grey) and *E. coli* Lipid A (black) at 1 μM peptide concentration in 10 mM Tris, pH 7.4 with additional 150 mM NaCl. (Paper II)

As shown in *Figure 11*, all peptides display binding to both LPS and its lipid A residue. Quantitatively, both the native GKY25 and its selected D-amino acid variant GKY25d adsorb much less at both LPS and lipid A than WFF25, with identical composition but with its amino acid sequence sorted according to hydrophobicity, thus displaying pronounced linear amphiphilicity (i.e., hydrophobic gradient along its chain). This higher linear amphiphilicity of WFF25 was found to result in higher peptide binding to both LPS and lipid A. Given the extensive binding to LPS and lipid A, the question arises which of these has higher binding affinity towards peptides. In this case, LPS binding for these peptides was compared to lipid A binding. LPS is likely to adsorb on the hydrophobic ($\theta \approx 90^{o}$) and negatively charged ($z \approx -40$ mV) methylated silica surface through its lipid A component forming a largely 3- dimensional adsorbed layer due to extension of the carbohydrate chains into bulk solution, while lipid A adsorption should form a two-dimensional surface due to the poor solubility of lipid A in aqueous solution. Thus, the effective surface areas of two systems are not comparable. Due to high LPS adsorption ($\approx$ 2.0 mg/m^2), the lipid A component will therefore be at least partly screened by the LPS carbohydrate chains, hence not fully accessible to peptide binding. Expectedly, *Junkes et al.* demonstrated previously that LPS carbohydrate chains have a larger number of potential peptide binding sites than lipid A[108].

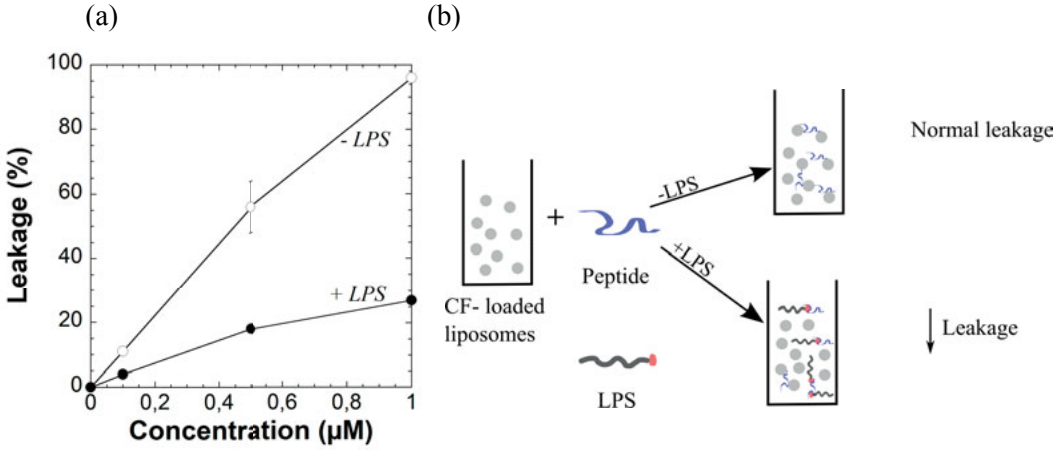
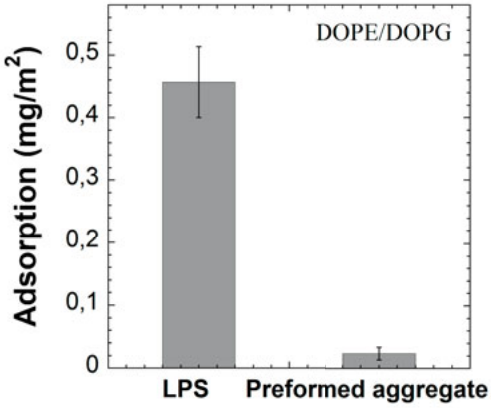


Figure 12. (a) GKY25-induced leakage of DOPE/DOPG (75/25 mol/mol) liposomes in the absence (open) and presence (filled) of 0.02 mg/ml *E. coli* LPS. Measurements were performed in 10 mM Tris, pH 7.4. (b) Schematic illustration of how LPS addition reduces peptide-induced liposome leakage. (Paper II)

Addressing further the issue of AMP binding preference, GKY25 binding to phospholipid membranes, to LPS and to its lipid A moiety was studied in greater details. While saturation binding of this peptide was comparable to anionic DOPE/DOPG bilayers, LPS and lipid A, reduced peptide-induced liposome leakage in the presence of LPS indicated that peptide binds preferentially to LPS over DOPE/DOPG membrane (*Figure 12*), in analogy to the results obtained for S1 peptidase –derived peptides (Paper I) and peptides derived from heparin cofactor II (Paper IV).

(a)



(b)

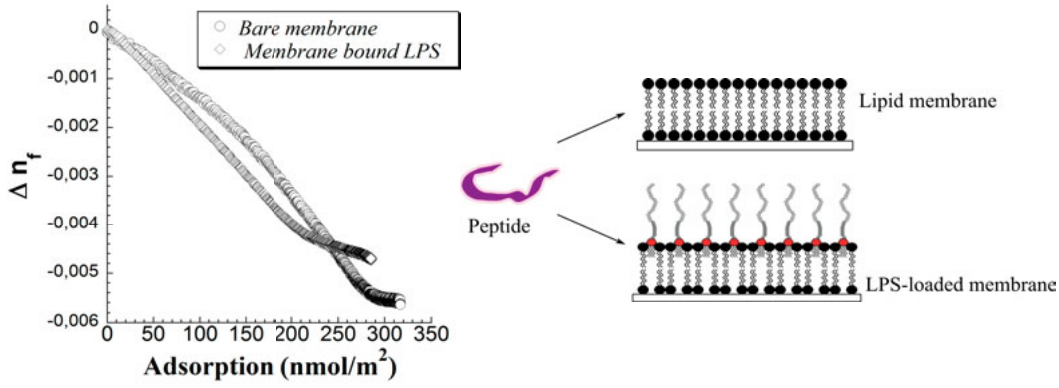


Figure 13. (a) Comparison of adsorption of LPS and preformed LPS-peptide aggregates (0.02 mg/ml LPS and 1 μM peptide) to DOPE/DOPG bilayer in 10 mM Tris, pH 7.4. To obtain preformed aggregates, LPS and peptide were mixed together 1 h before addition. (b) Comparison of GKY25-induced disordering of DOPE/DOPG membranes with (0.7 mg/m^2) and without LPS in 10 mM Tris, pH 7.4. (Paper II)

Furthermore, since binding of anionic LPS to anionic DOPE/DOPG membrane is hydrophobically driven exclusively by lipid A, preferential peptide binding to lipid A over LPS polysaccharide chains was demonstrated by the strongly reduced LPS binding to such membranes observed for peptide/LPS aggregate, as shown in *Figure 13a*. Obviously, in bacteria, LPS is incorporated into the anionic lipid membrane. Therefore, GKY25 binding was investigated also for DOPE/DOPG bilayers containing LPS. As can be seen in *Figure 13b*, GKY25 causes similar membrane destabilization, irrespective of the presence of LPS and binds to almost the same extent in both cases, inserting continuously during adsorption with no threshold for membrane incorporation. As the lipid A moiety is incorporated into the negatively charged lipid bilayer, peptide interaction with this component can only occur on peptide penetration into the bilayer. Through the combined binding and leakage experiments, GKY25 was thus found to have highest binding affinity for lipid A, both when adsorbed at methylated silica and when incorporated into the lipid bilayer. Together with peptide binding to LPS, these competitive binding experiments demonstrated peptide binding affinity to follow the order lipid A>LPS> lipid membrane. Similarly, *Yang et al.* found a higher peptide affinity for lipid A binding of rALF-Pm3[109], while *Brandenburg et al.* demonstrated that lactoferrin binds preferentially in the proximity of the phosphate groups of lipid A[94]. This peptide binding preference seems to be dependent on AMPs investigated, as *Junkes et al.* found the binding of cyclic R/W- rich peptides to decrease on removal of the O-antigen and outer polysaccharides from LPS[108]. In addition to this, it should be here mentioned that AMPs binding to the anionic LPS polysaccharide domain is expected to lead osmotic deswelling, as commonly observed in other polyelectrolyte systems[110]. Despite this, AMPs are able to penetrate LPS layers to access lipid A moiety.

Effect of linear amphiphilicity on membrane interaction

For the same GKY25 peptide variants, effects of linear amphiphilicity on membrane interactions were investigated. As shown in *Figure 14a*, WFF25 (with retained composition but increased linear amphiphilicity) displays higher membrane binding and thus, resulting higher peptide-induced liposome rupture (*Figure 14b*) than native GKY25 and GKY25d (suppressed helix-related amphiphilicity) for anionic lipid membranes. As shown in *Figure 14c*, however, GKY25 displays more potent antimicrobial activity for Gram-negative *E. coli* and Gram-positive *S. aureus*. Thus, GKY25 is more potent than GKY25d both in liposome destabilization and antimicrobial effects, emphasizing the contribution of (amphiphilic) helix formation for membrane destabilization for these peptides. Strikingly, however, WFF25 induces the *weakest* bacterial killing, despite this peptide displaying the

highest binding to, and destabilization of, anionic DOPE/DOPG ("bacteria mimicking") membranes.

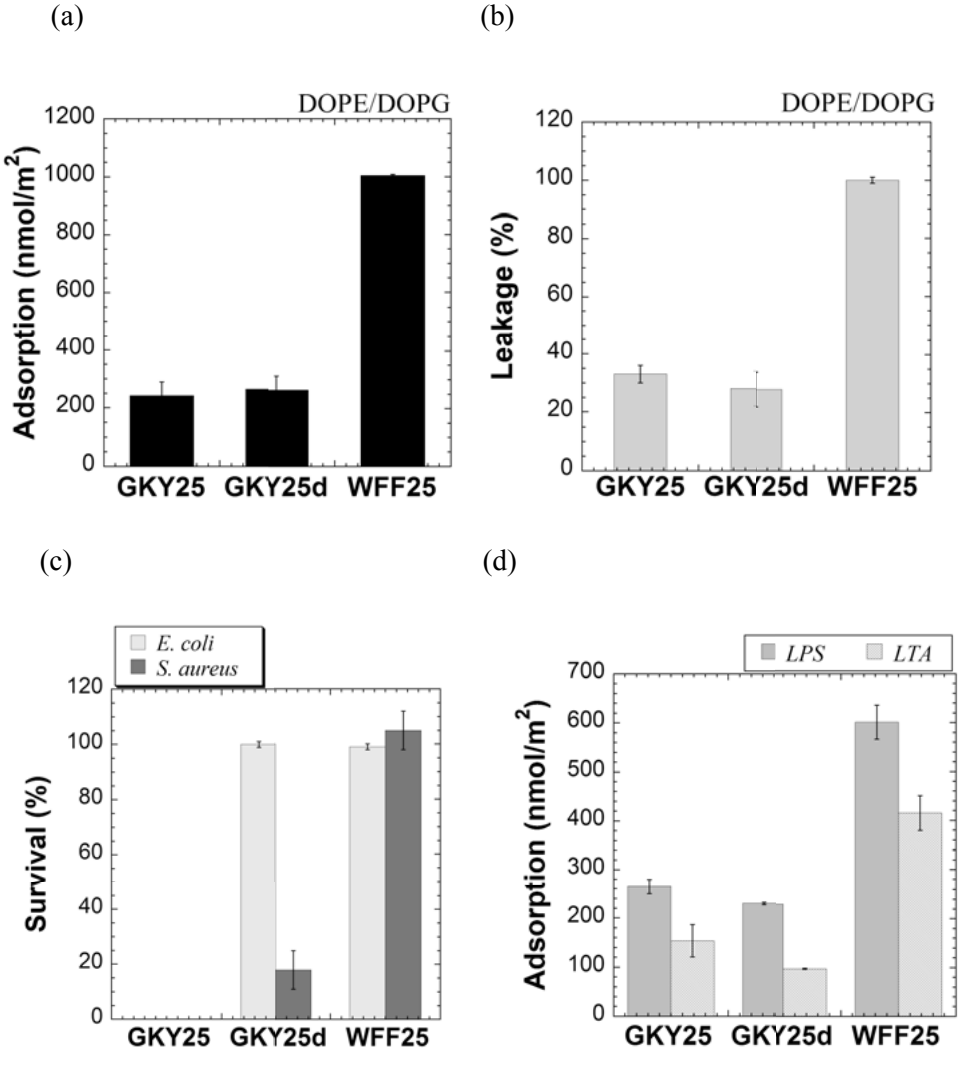


Figure 14. (a) Peptide binding to supported DOPE/DOPG bilayers in 10 mM Tris, pH 7.4, with additional 150 mM NaCl. (b) Peptide –induced liposome leakage for DOPE/DOPG (75/25 mol/mol) in 150 mM NaCl, 10 mM Tris, pH 7.4. (c) Antimicrobial activity of peptides (30 μM) against *E. coli* (grey) and *S. aureus* (black) as determined by viable count assay in 10 mM Tris, pH 7.4, with additional 150 mM NaCl. (d) Peptide adsorption to *E.coli* LPS and *S. aureus* LTA in 10 mM Tris, pH 7.4, with additional 150 mM NaCl. (Paper III)

Importantly, it was found that peptide–induced liposome flocculation/fusion does not contribute to the membrane disruption of liposomes at this concentration (1 μM) despite of WFF25 adsorbs strongly at anionic lipid membrane. Thus, the discrepancy between results obtained in anionic bacteria-mimicking model membranes known to correlate with antimicrobial effects[111-114] instead, this seems to originate from the bacterial membranes contain considerable amount of non-lipid components not present in the model liposomes, notably LPS in Gram-negative and LTA in Gram–positive. Thus, WFF25 binds extensively not only to LPS, but also to LTA (*Figure 14d*) due to its pronounced linear amphiphilicity. Particularly as WFF25 is sufficiently amphiphilic to self-assemble into aggregates (Paper III), LPS/LTA seems to be able to scavenge WFF25, preventing it from reaching a sufficient concentration at the membrane to achieve efficient membrane disruption. In addition to such direct scavenging, preferential peptide (aggregate) binding to LPS and LTA may also cause formation of an effectively impermeable layer through osmotic deswelling, in line to the effects previously demonstrated by polyelectrolytes while interacting with oppositely charged peptides[110, 115]. Taken together, these results show an unusual discrepancy between antimicrobial effects and liposome leakage due to pronounced effects of linear amphiphilicity for WFF25.

Peptide-induced LPS binding to mammalian membrane

In an effort to further clarify potential mode of action of peptide-induced anti-inflammatory effect, a series of peptides derived from human heparin cofactor II (paper IV) were investigated as shown in *Table 2*.

Table 2. *Sequence and key properties of peptides investigated. (Paper IV)*

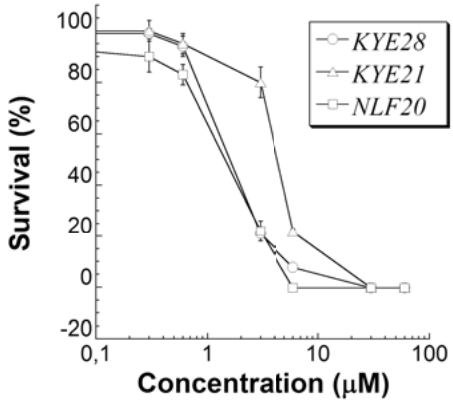
Peptide	Sequence	Z_{net}[1] *(pH 7.4)*	H[2]
KYE28	KYEITTIHNLFRKLTHRLFRRNFGYTLR	+6	-0.72
KYE21	KYEITTIHNLFRKLTHRLFRR	+5	-0.79
NLF20	NLFRKLTHRLFRRNFGYTLR	+6	-0.80

[1]Z_{net}: net charge; [2]H mean hydrophobicity

In order to investigate structure-activity-relationship, the full length peptide KYE28 was compared to two truncated variants, i.e., KYE21 and NLF20. As shown in *Figure 15*, KYE28 displays potent antimicrobial and anti-inflammatory effects whereas KYE21 retains both the antimicrobial and anti-inflammatory activity of KYE28 (but both attenuated), while NLF20 displays substantially reduced anti-inflammatory effect but maintained antimicrobial effect. The anti-inflammatory effect thus decreases in the order

KYE28>KYE21>NLF20. Despite this, these peptides bind to a similar extent to both LPS and lipid A.

(a)



(b)

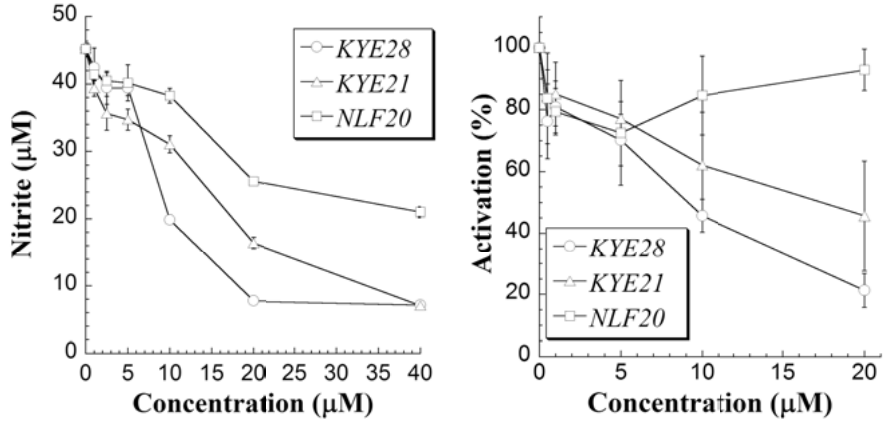


Figure 15. Antimicrobial effect, as determined by viable count assay in 10 mM Tris, pH 7.4, with additional 150 mM NaCl against Gram-negative *E. coli*. (b) Effect of the indicated peptides on macrophages. Macrophages were incubated with LPS in presence of peptides, followed by monitoring of NO production or NF-κB activation, respectively. (Paper IV)

Despite these differences in anti-inflammatory effects, the binding of these three peptides to LPS and lipid A was quite similar, clearly excluding LPS/lipid A-binding as the single anti-inflammatory mechanism. Addressing this, we speculated that these peptides, due to their charge and amphiphilicity, could induce localized LPS scavenging at mammalian cell membranes. Since such cells (e.g., monocytes and macrophages) are rich in zwitterionic lipids and therefore carrying a low negative charge, adsorption of cationic peptides may induce a net positive charge, thus facilitating

electrostatically driven binding of anionic LPS to these membranes. Demonstrating this, *Figure 16a* shows that binding of KYE28 variants to DOPC/cholesterol (mammalian mimicking) membranes induce a net positive potential of the membrane, thus LPS binding to the peptide containing zwitterionic membrane increases (*Figure 16b*).

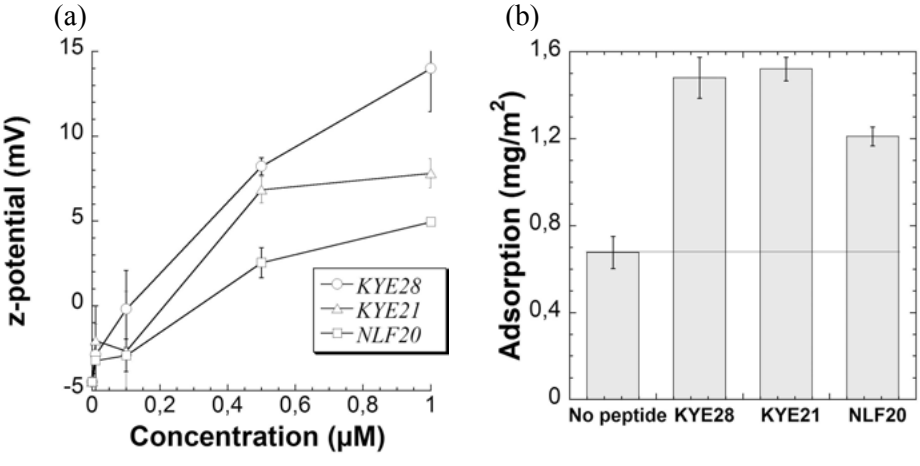


Figure 16. (a) z-potential of DOPC/cholesterol (60/40 mol/mol) liposomes as a function of peptide concentration in 10 mM Tris, pH 7.4. (b) LPS adsorption at DOPC/cholesterol (60/40 mol/mol) supported bilayer with and without peptide preadsorption (peptide concentration 1 μM). Measurements were performed in 10 mM Tris, pH 7.4. (Paper IV)

Peptide-induced indirect LPS binding results membrane-localized LPS scavenging may cause avoidance of the LPS-triggered NF-κB activation reaction through promoting an alternative binding site for LPS to macrophage/monocyte membranes, thus provides a potential anti-inflammatory mechanism. Indeed, as seen in *Figures 15b and 16b*, NLF20 with least anti-inflammatory effect displays weakest capacity of such localized LPS scavenging. However, KYE28 and KYE21 are quite comparable with regard to membrane–localized LPS binding, despite KYE28 displaying more efficient anti-inflammatory effects. Therefore, it seems like peptide-induced LPS scavenging localized at mammalian cell membrane may contribute partially to the anti-inflammatory effect of KYE28 variants, but is not the sole mechanism involved.

Peptide effect on LPS aggregate structure

In the classical pathway, LPS triggers inflammation cascade through lipid A-mediated binding to lipopolysaccharide-binding protein (LBP) at macrophage surfaces, recognized by CD14 and Toll-like receptor 4 (TLR4),

subsequently results in an up-regulation of NF-κB and pro-inflammatory cytokines [75]. In addition, however, LPS aggregates have been found to play an important role in inflammation triggering[67]. Hence, peptide-induced LPS aggregates disruption can potentially be related to anti-inflammatory effects. In line with this, *Rosenfeld et al.* demonstrated peptide-induced LPS aggregate fragmentation by LL-37 as well as two synthetic 15-mer K/L peptides, also displaying anti-inflammatory effects[116]. Similar findings were reported by *Bhunia et al.* for fowlicidin-1 fragments[117] and by *Mangoni et al.* for temporin variants[118].

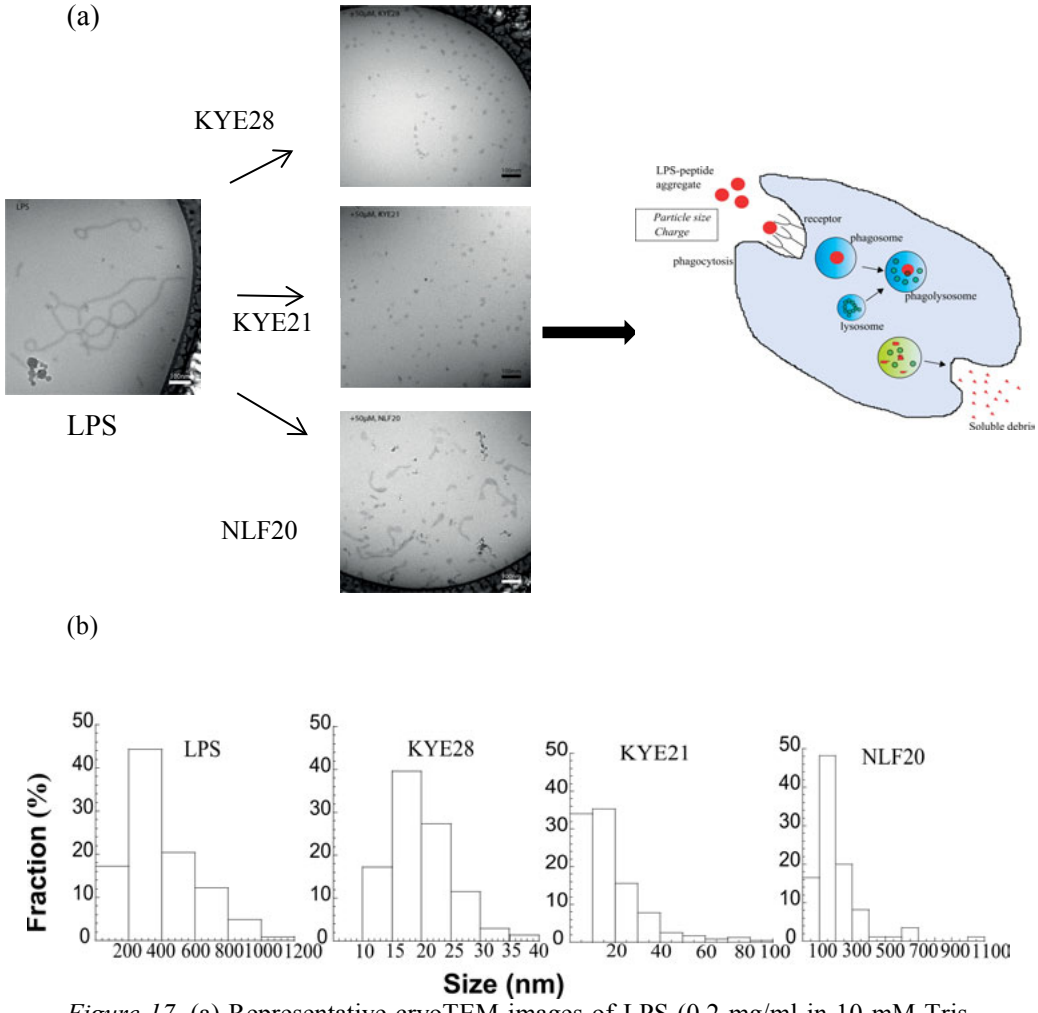


Figure 17. (a) Representative cryoTEM images of LPS (0.2 mg/ml in 10 mM Tris, pH 7.4) in the absence and presence of KYE28, KYE21 and NLF20 (50 μM) together with a schematic illustration of size-dependent phagocytosis. (b) Histograms showing the size distributions for different systems are shown. (Paper IV)

Addressing the issue of peptide-induced disruption of LPS aggregates, heparin cofactor II peptides were further investigated (Paper IV). In doing so, cryoTEM showed that the effects of these peptides on LPS aggregate structure correlate with their anti-inflammatory potency. As shown in *Figure 17a*, KYE28 causes disintegration of LPS aggregates initially to short linear fragments and subsequently form small dense spherical particles at higher peptide concentration. Most likely, this peptide-induced LPS fragmentation and densification is due to a reduction of electrostatic repulsion between negatively charged LPS carbohydrate chain through charge neutralization, as well as peptide binding to lipid A phosphate group, thereby facilitating denser packing, as also suggested by ANS fluorescence results. In comparison, KYE21 is less efficient in fragmenting LPS aggregates, resulting in larger spherical particles as shown in *Figure 17b*. Finally, NLF20 is least efficient in rupturing and compacting LPS aggregates. The ability of AMPs to cause LPS aggregates disruption and densification thus seems to be important for their anti-inflammatory effect.

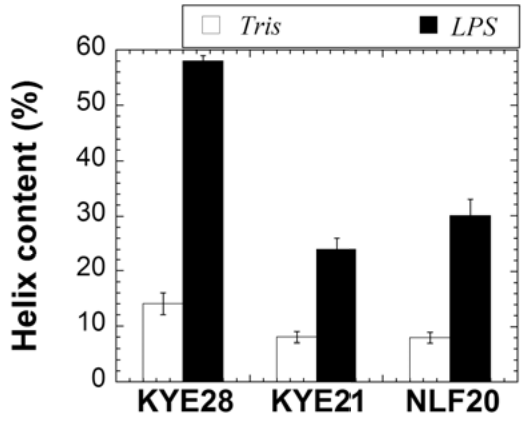


Figure 18. Effective helix content of the peptides in the presence of *E. coli* LPS (0.2 mg/ml). (Paper IV)

Furthermore, effect of peptide secondary structure on LPS interaction was studied for same peptides. On LPS binding, there was pronounced induction of helix formation for KYE28, but considerably less so for KYE21 and NLF20 (*Figure 18*). A similar correlation between anti-inflammatory effect and ordered structural transition in the LPS/peptide aggregates was previously reported for melittin[119], S1 peptidase peptides (paper I), and thrombin-derived peptides (paper II). Taken together, cryo-TEM results indicate that the order transition of lipid A on LPS binding was correlated to prominent packing reorganizations (disintegration/ densification) of LPS aggregates, which CD indicates were facilitated by peptide secondary structure transition. The resulting reduction in size and negative charge of LPS is expected to facilitate

phagocytosis, in analogy to size and charge dependence of phagocytosis of other types of nanoparticular systems[120]. Through this, an alternative pathway to LPS-LBP/CD14 binding/activation is provided, resulting in attenuation, or even blocking, of the inflammatory activation occurring in the absence of peptide. Indeed, such phagocytosis-related scavenging as anti-inflammatory response has been previously observed, although for inflammation caused by amyloid Aβ rather than by LPS. Thus, *Richman et al* investigated protein-microspheres with an Aβ–recognizing peptide, and were able to correlate anti-inflammatory effects of the latter with triggering of Aβ phagocytosis, thereby avoiding the alternative triggering pathway[121]. Along the same line, the cationic peptide LL-37 has been demonstrated to transfer complexed negatively charged molecules into cells, which has been used, e.g., to transfect eukaryotic cells[122].

PEGylation for enhanced AMP performance

PEGylation i.e. conjugation with poly(ethylene)glycol (PEG) has been extensively investigated as an approach to increase performance for a range of peptide therapeutics through offering a series of potential advantages including reduction in serum protein adsorption[123], increased bloodstream circulation time, reduced uptake in tissues related to the reticuloendothelial system [124], increased resistance to proteolytic degradation as well as reduced aggregation, toxicity and immune response[125]. It has been previously reported that PEGylation offers reduced toxicity and proteolytic susceptibility of AMPs but at a cost of reduced antimicrobial effect[126, 127].

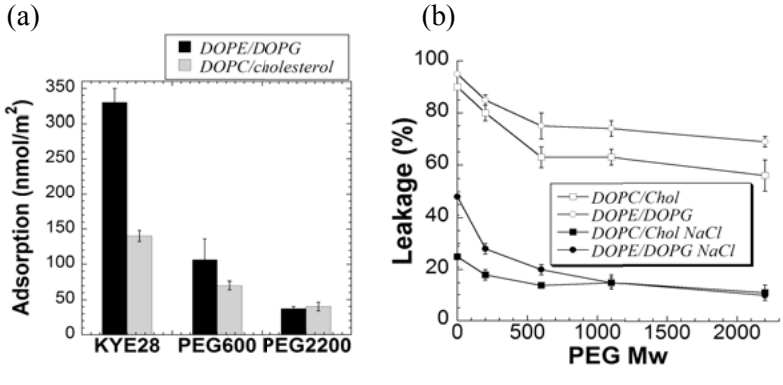


Figure 19. (a) Peptide binding to DOPE/DOPG (75/25 mol/mol) and DOPC/cholesterol (60/40 mol/mol) bilayers in 10 mM Tris, pH 7.4 ("PEGMw" refers to "KYE28PEGMw" throughout). (b) Peptide-induced leakage induction of DOPE/DOPG (75/25 mol/mol) and DOPC/cholesterol (60/40 mol/mol) liposomes. Measurements were performed in 10 mM Tris, pH 7.4, with or without 150 mM. (Paper V)

Considering this, we were interested in how PEGylation affected anti-inflammatory properties of peptides. In doing so, effect of PEG length and localization on membrane as well as LPS interaction was further investigated for KYE28. As shown in *Figure 19a*, PEGylation results in a drastic reduction in amount of peptide binding for both DOPE/DOPG ("bacteria-mimicking") and DOPC/cholesterol ("mammalian mimicking") bilayers in an Mw-dependent manner. The decreased binding displayed for longer PEG conjugates was largely due to the non-adsorption of PEG at these membranes.

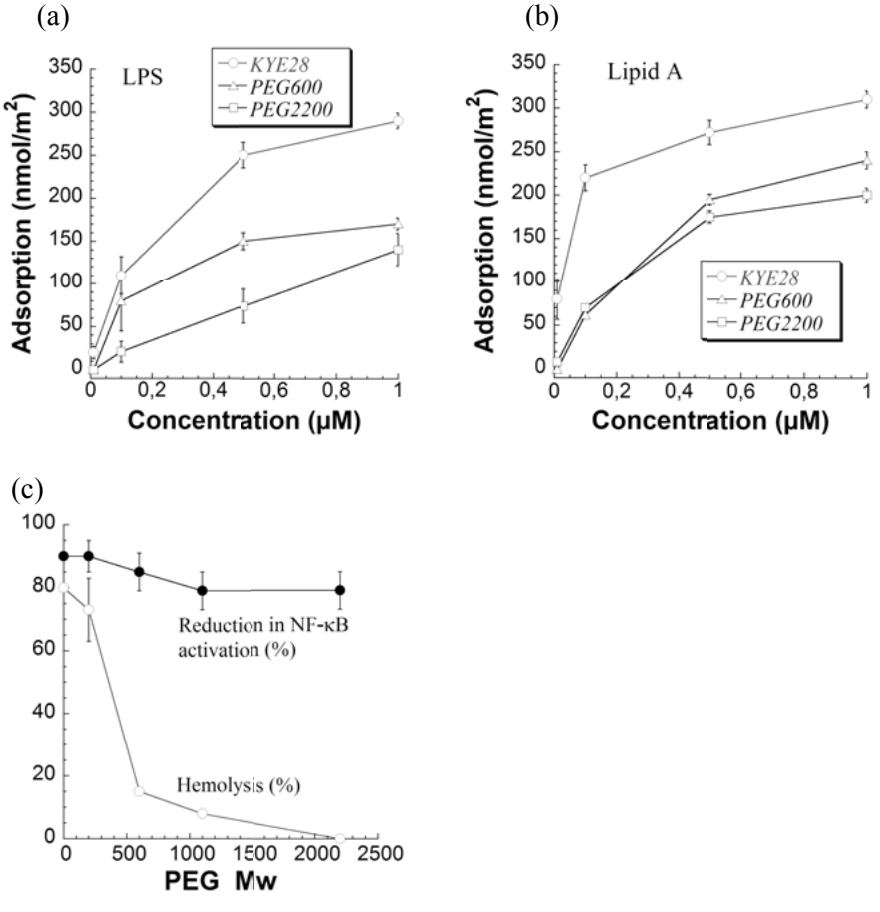


Figure 20. Peptide binding to (a) *E. coli* LPS and (b) *E. coli* lipid A. Measurements were performed in 10 mM Tris, pH 7.4. (c) Effect of PEG length on hemolysis and anti-inflammatory effect of PEGylated KYE28. For the latter, RAW-blue macrophages were incubated with *E. coli* LPS in presence of peptides at the indicated concentration, followed by monitoring of NF-κB activation. (Paper V)

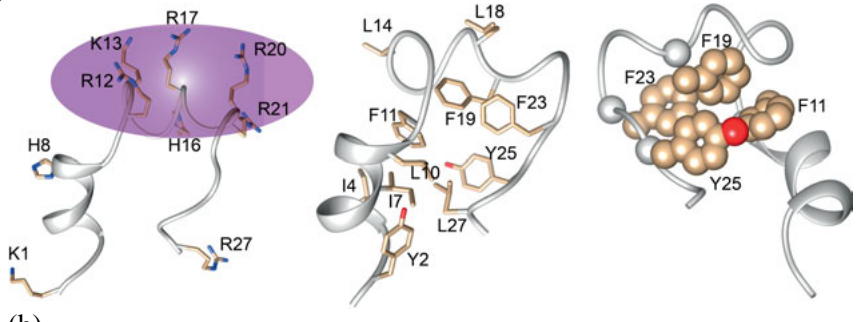
As a result of reduced peptide binding, the corresponding peptide-induced liposome leakage also decreases (*Figure 19b*), indicating a suppression of KYE28 antimicrobial potency in an Mw-dependent manner. In addition, a decrease in hemolysis was observed with increasing PEG length (*Figure 20c*). Through the latter, conditions could be found, at which the PEGylated peptide display efficient antimicrobial activity, but at same time causing null hemolysis.

Furthermore, in analogy to the reduced membrane binding of the PEG conjugates, reduction in LPS and lipid A binding was observed with PEG length (*Figure 20a-b*). Interestingly, Peptide ability to block LPS-induced NF-κB activation reaction and thus, anti-inflammatory properties largely retained (*Figure 20c*). Consequently, PEG conjugation seems to offer opportunities in the development of effective and selective anti-inflammatory peptides through optimizing the performance of anti-inflammatory peptides e.g. reduced toxicity and increased proteolytic stability.

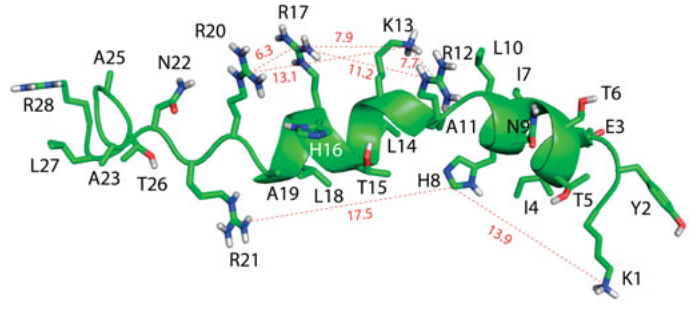
Role of aromatic residues in LPS interaction

It order to further investigate peptide-induced secondary structure transition in peptide/LPS complexes, as well as their relation to anti-inflammatory effects, LPS interactions of KYE28 was further investigated by NMR (Paper VI).

(a)



(b)



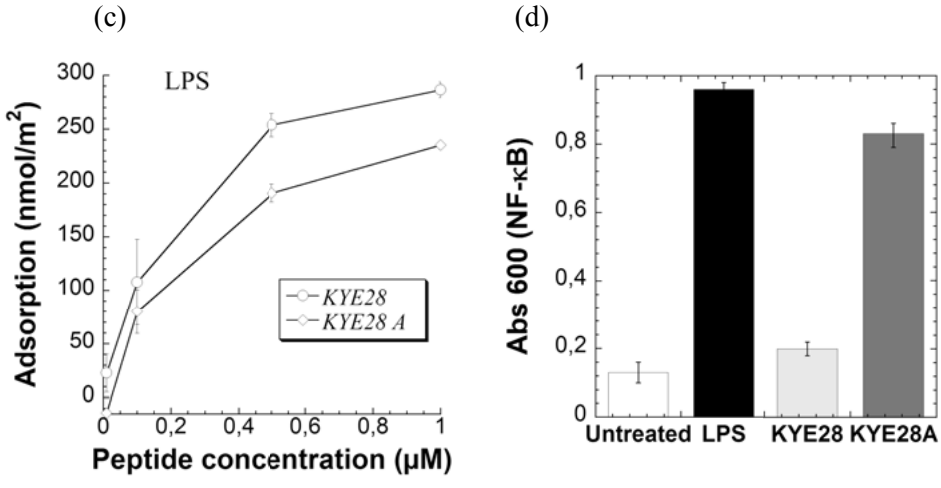


Figure 21. (a) Representative structure of the LPS-bound form of KYE28, showing the orientation of (left panel) positive charges forming a cationic face highlighted in violet and (middle panel) the hydrophobic residues forming an inner core through interaction between its side chains thus adopting an amphipathic orientation. (Right panel) representing the aromatic zipper composed of F11, F19, F23 and Y25, highlighted in spheres which drives the stabilization of the folded conformation of KYE28 in LPS. (b) Representative structure of KYE28A in LPS showing the orientation of the positive charges over one side of the two helices. The hydrophobic residues remain oriented towards the other end maintaining an amphipathic orientation. (c) Binding of KYE28 and KYE28A to *E. coli* LPS in 10 mM Tris, pH 7.4. (d) Effects of the indicated peptides on macrophages. RAW264.7 macrophages were incubated with *E. coli* LPS in presence of peptides (20 μM), followed by monitoring of NF-κB activation, respectively. (Paper VI)

Through this, three-dimensional solution structure of KYE28 in LPS was studied to understand the key residues involved in stabilization of its adopted conformation, as well as to provide the knowledge on structure-activity correlation. In particular, NMR studies revealed that aromatic residues of KYE28, particularly F11, F19, F23 and Y25, formed an aromatic zipper on LPS binding, and played an important role in stabilizing the amphiphilic structure of KYE28 in LPS (*Figure 21a*). Similar results were previously reported for 16-residue AMP derived from dengue viral fusion peptide[128], paraxin, and the designed peptide MSI-594[129, 130]. In addition, positively charged Arg and Lys residues remain flexible and oriented at one particular face, forming a polar exterior shell that may possibly interact with the negatively charged phosphate and carboxyl head groups of LPS. To further demonstrate the importance of aromatic packing interactions, a mutant peptide (KYE28A), having 4 Ala mutations (A11, A19, A23 and A25), was compared to native KYE28. In contrast to KYE28, KYE28A adopted an open helical structure in LPS where aromatic packing interactions were

completely lost (*Figure 21b*). Furthermore, KYE28A was shown to display attenuated anti-inflammatory effect, as well as lower LPS binding capacity compared to KYE28 (*Figure 21c-d*). Thus, the aromatic zipper of KYE28 plays a crucial role in LPS interaction and potentially contributes to its anti-inflammatory activity.

Conclusions

The results presented in this thesis demonstrate the importance of AMP binding to inflammatory agents such as lipopolysaccharide (LPS) in Gram-negative bacteria and lipoteichoic acid (LTA) in Gram-positive bacteria, to the lipid A moiety in LPS, for anti-inflammatory effect of AMPs. Furthermore, the results also show that how physiochemical properties of AMP (charge, hydrophobicity, length and peptide secondary structure) influence their interactions with both lipid membrane and non-lipid membrane components, such as LPS, lipid A and LTA, as well as the consequence of this interaction for antimicrobial and anti-inflammatory effects of these AMPs.

Specially, results showed that peptides bind to LPS through a combined effect of peptide net charge and hydrophobicity. Importantly, peptide binding to LPS and lipid A was found to be necessary for anti-inflammatory effect of AMPs investigated. However, anti-inflammatory effects of AMPs do not depend simply on the amount of peptide bound to LPS or its lipid A moiety. Instead, anti-inflammatory effects were found to be of complex origin, and through several different mechanisms. In addition to direct LPS/lipid A binding, peptide-induced LPS binding to membranes of human cells was demonstrated to provide a potential anti-inflammatory mechanism through membrane localized LPS scavenging and thus, avoiding NF- κB activation. In this context, some correlation was observed for heparin cofactor II peptides between peptide-induced membrane potential increment, subsequent LPS binding, and anti-inflammatory effects. In addition, peptide-induced fragmentation and densification of LPS aggregates provides a likely additional mechanism through controlling the fraction of lipid A exposed and accessible to CD14/TLR4 binding, thus deflecting the NF-κB activation pathway. In this respect, a correlation between AMP anti-inflammatory effects and their effects on LPS aggregates disruption was found, suggesting the peptide-induced phagocytosis.

Furthermore, studies examined the PEGylation of one of these peptides for binding to lipid membranes, LPS and lipid A, as well as implications for antimicrobial activity, cellular toxicity, and anti-inflammatory effect. Through these studies, it was demonstrated that PEGylation results in reduced surface activity and antimicrobial effect with increasing length of the PEG chain, but simultaneously also in reduced toxicity. Importantly, PEGylation does not lead to any significant decrease in the peptide's anti-

inflammatory effect, opening opportunities for peptides with low toxicity and for peptides with reduced but "*sufficient*" antimicrobial activity with maintained anti-inflammatory effect.

Furthermore, structural aspects of LPS-AMP interaction were studied demonstrating potential importance of aromatic zipper for anti-inflammatory effect of AMPs. In particular, aromatic packing interactions contribute to helical structure stabilization of peptide on LPS binding whereas the positively charged residues participate in forming an outer polar surface/shell of LPS/peptide complexes.

Taken together, results presented in this thesis provide knowledge on important prerequisites/factors affecting the anti-inflammatory activity of peptides, as well as their antimicrobial effect, which can be considered for developing these AMPs as effective drug therapeutics for the treatment of inflammatory diseases.

Development outlook

The work included in thesis is directed towards biophysical aspects of AMP-LPS interaction in order to further clarify the details of this interplay and their implications for antimicrobial and anti-inflammatory effects. In this respect, mode- of- action of such AMPs was found to be complex involving multiple molecular mechanisms. The most important of these seem to be:

1. Direct LPS scavenging: Binding of peptide to LPS prevents its binding to LBP through blocking the lipid A epitope of LPS, thus avoiding the activation of inflammatory cascade named NF-κB pathway.

2. Indirect membrane –localized LPS scavenging: positive potential build-up of membrane of monocytes/macrophages through peptide adsorption, in turn causing binding of anionic LPS.

3. Peptide-induced phagocytosis through LPS aggregate disruption

Although some support for these mechanisms were obtained from the work in this thesis, they remain somewhat speculative and require further experimental support. For example, membrane-localized LPS scavenging needs to be further investigated for human cells (as opposed to model lipid membranes) in relation to factors affecting peptide binding and positive membrane potential build-up on different human cells including macrophages/monocytes. Furthermore, mechanism involved in trafficking of amphiphilic peptides and their LPS complexes in macrophages needs to be clarified together with the interplay between properties affecting peptide/LPS aggregate (size and charge) and cell internalization through phagocytosis. Further work is also needed on factors affecting LPS-LBP binding, which can be done, e.g., by studying binding of preformed peptide/LPS aggregates binding to surface-bound LBP, or by studying downstream complexes with membrane-bound TLRs. Ideally, adsorption of LPS/peptide complexes should also be done to intact membranes of macrophages. Apart from direct receptor binding, possibly followed by endocytosis, some cationic peptides have been reported to be taken up by mammalian cells through membrane internalization specifically,

translocation[131] that requires further investigation. In addition, future studies also need to pay attention to the ability of AMPs against inflammation triggered by other inflammation-triggering compounds, such as LTA in Gram-positive bacteria and zymosan in fungi, in order to differentiate between system- specific and general effects.

From a methodological perspective, there are also needs of further development. For example, while investigating antimicrobial effect of the highly amphiphilic peptide WFF25, bacterial killing or bacterial lysis was found not to be in agreement with lysis of "bacteria-mimicking" liposome membranes due to their pronounced LPS/ LTA binding that results dramatically reduced antimicrobial effect through peptide scavenging. Thus, for highly amphiphilic peptides, simple model lipid systems such as liposomes (also those formed by bacteria lipid extracts) seem not to present a good model for bacteria. For such peptides, methods for stratification or deposition at surfaces may therefore be a useful tool with a higher degree of biomimickry.

Popular scientific summary

Infectious diseases cause millions of death each year and result in tremendous socioeconomic costs. One main reason behind this is the rapid growth of bacterial resistance against conventional antibiotics. Due to increasing antibiotic resistance, there is an urgent need to identify novel approaches to treat bacterial infections. In this context, small proteins called antimicrobial peptides (AMPs) offer great opportunities. AMPs attack membrane of different microorganisms (bacteria, fungi) and cause membrane rupture that results in bacteria killing. AMPs contain positive charges and hydrophobic residues, making them ideal candidate to disrupt bacterial membranes, which are negatively charged. Peptides investigated for current work display both antimicrobial and anti-inflammatory effects. These host defense AMPs have attracted special interest as potential therapeutics against both infections and resulting inflammation particularly for sepsis. These diseases are characterized by bacterial infection together with acute inflammation that needs to be treated successfully. There is urgency to find effective and safe drugs due to unavailability of such drugs in the market, presently resulting in 30-40% mortality (≈70% for elderly and chronically ill patients) in the industrialized world, and considerable more than this in developing countries. While, it has been extensively investigated that AMPs kill bacteria through direct membrane disruption, now the question is that what would be the mechanism behind the anti-inflammatory effects of host defense AMP. Thus, anti-inflammatory effect of AMPs was believed to be related to their interactions with inflammatory substances such as lipopolysaccharide (LPS) in Gram-negative bacteria and lipoteichoic acid (LTA) in Gram-positive bacteria. In this regard, single component based model systems for LPS, lipid A and LTA were developed. It has been shown that host defense AMPs bind extensively to LPS with a combined effect of peptide net charge and hydrophobicity. LPS and lipid A binding was compared to anti-inflammatory effect of AMPs. It was found that LPS/lipid A binding to AMPs is necessary for anti-inflammatory effect. However, amount of LPS/lipid A binding was not quantitatively correlated to the anti-inflammatory effect. Instead, it was found that the anti-inflammatory effect of AMPs is complex and caused by several different mechanisms, i.e.,

1. Direct LPS scavenging: Binding of peptide to LPS prevents its binding to LBP through blocking the lipid A epitope of LPS, thus avoiding the activation of inflammatory cascade named NF-κB pathway.

2. Indirect membrane –localized LPS scavenging: positive potential build-up of membrane of monocytes/macrophages through peptide adsorption, in turn causing binding of anionic LPS.

3. Peptide-induced phagocytosis through LPS aggregate disruption

Furthermore, since AMPs are sensitive to proteolytic degradation, and also cleared from bloodstream circulation rapidly, poly (ethylene) glycol (PEG) conjugation was used to increase performance of these peptides. It was found that PEGylation of these peptides results in decreased antimicrobial potency but was able to effectively kill bacteria. Interestingly, while PEGylated peptide displays reduced LPS/lipid A binding and its capacity to disrupt LPS aggregates, its anti-inflammatory property was largely retained. Thus, PEGylation may offer an interesting approach to improve the conditions of anti-inflammatory peptides e.g. reduced toxicity with increased stability.

In summary, results presented in the thesis provide knowledge on important prerequisites/factors affecting the anti-inflammatory activity of peptides, as well as their antimicrobial effect, which can be considered for developing these AMPs as effective drug therapeutics for the treatment of inflammatory diseases.

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Shalini,
Uppsala, April 2016

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